W.P. Arnold

Induction and modulation of ornithine decarboxylase activity in human epidermis

Induction and modulation of ornithine decarboxylase activity in human epidermis

Induction and modulation of ornithine decarboxylase activity in human epidermis

Een wetenschappelijke proeve op het gebied
van de Medische Wetenschappen

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen
op donderdag 10 februari 1994
des namiddags te 3.30 uur precies
door

WIM PETER ARNOLD

geboren op 3 december 1961 te Arnhem

Promotor: Prof. Dr. Dr. P.C.M. van de Kerkhof

Co-promotor: Dr. J. Schalkwijk

copyright: W.P. Arnold, Nijmegen 1994
drukwerk: Grafisch Service Centrum Van Gils B.V., Wageningen
omslag: S.R. Velde

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Arnold, W.P.

Induction and modulation of ornithine decarboxylase activity in human epidermis / W.P. Arnold. - [S.l. : s.n.]. - Ill.
Thesis Nijmegen. - With index, ref. - With summary in Dutch.
ISBN 90-9006606-3
Subject headings: onithine decarboxylase / human epidermis.

Chapter I

INTRODUCTION

1.1 General introduction

1.1.1 Epidermal growth and differentiation

The subject of investigation in this thesis is human epidermis *in vivo*. In the most general sense, the function of skin is to seperate the body from its environment; that is, to keep the inside in and the outside out. This task is partly accomplished by the dermis ("mechanical barrier"), melanocytes ("radiological barrier") and Langerhans cells ("immunological barrier"), but the "chemical defence" is formed exclusively by keratinocytes. They make up three distinct, successive compartments: an innermost layer of dividing keratinocytes (stratum basale), an intermediate region of differentiating keratinocytes (stratum spinosum & stratum granulosum) and a superficial layer of cornified envelopes (stratum corneum). It is this last layer which is the true barrier: almost totally impermeable to water, salts and micro-organisms, partly impermeable to chemicals and ultraviolet light and continuously renewed from the inside to compensate for natural wear and tear. Hence epidermal growth and differentiation are closely linked. Figure 1 shows the histology of healthy human epidermis.

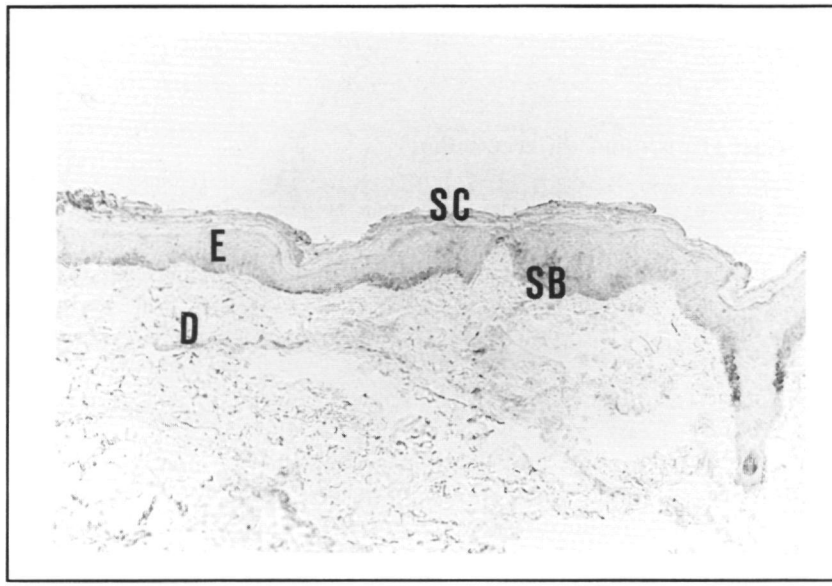

Fig.1 **D**= dermis, **E**= epidermis, **SB**= str. basale, **SC**= str. corneum

1.1.2 Epidermal growth in relation to cutaneous inflammation

The physiological process of continuous epidermal renewal mentioned in paragraph 1.1.1 can become pathologic in several ways, resulting in the richly scattered palette of dermatology. Almost all skin diseases, however, are characterized by variable degrees of disturbances in proliferation and differentiation only. Besides, proliferation and differentiation are nearly always related to inflammation, primarily or secondarily. These are the same processes that are involved in the response to a trauma. Removal of the stratum corneum by tape stripping, irradiation with UV-B, application of phorbol esters or

allergens: sooner or later this will be followed by hyperproliferation, inflammation and altered differentiation. Hence a common pathway seems likely; an hypothetical schedule is given in Figure 2.

For example, a somatic mutation will cause an "intrinsic error" in the biochemical processes controlling proliferation. The mutant cell will divide and divide without control and after a critical level the tumor will cause pressure and diminished bloodflow to its surrounding cells. This can be interpreted as a trauma, resulting in inflammation and disturbed differentiation of both the tumor and its environment. But in the monogenetic disorders of keratinization an "intrinsic error" dysregulates a specific part of the biochemical process controlling differentiation. As explained in paragraph 1.1.1, this will result in a decreased barrier function and hence any minor stimulus from outside may act as a trauma. Of course this will be followed by hyperproliferation and inflammation. In an experimental approach, it is also possible to induce inflammation "en sec". Five to fifty ng of LTB_4 applied to the skin will cause an infiltrate of polymorphonuclear leucocytes. This "aggressive" micro-abcess will be very traumatizing for its normal neighbour cells, finally resulting in hyperproliferation and pathologic differentiation.

In the common pathway described in Figure 2, protein kinase C (PKC) probably plays a role. In the epidermis, stimulation of the membrane-bound epidermal growth factor receptor (with intrinsic tyrosine kinase activity) by transforming growth factor α (an endogeneous cytokine) causes activation of phospholipase C. This enzyme in turn causes the formation of diacylglycerol, which finally activates PKC.[1] Activated PKC will then 1) induce proliferation, i.e. by phosphorylation (and activation) of transcription factors, and 2)

will initiate inflammation by phosphorylation (and hence inactivation) of lipocortin. The former leads to the production of ornithine decarboxylase,[2] which is essential for new DNA synthesis, and the latter, via release of phospholipase A_2, leads to the formation of leucotrienes and prostaglandins from arachidonic acid.[3] Both are potent mediators of inflammation; prostaglandin E_2 causes vasodilatation and leucotriene B_4 attracts inflammatory cells such as polymorphonuclear leucocytes. Hence, in pathologic circumstances, epidermal growth and inflammation are closely linked, both at the clinical and biochemical levels.

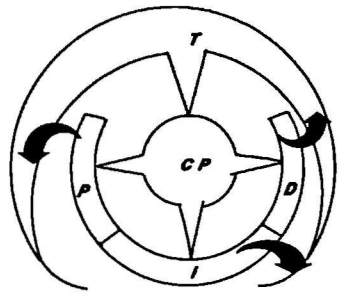

Fig.2 **CP**= common pathway, **D**= differentiation, **I**= inflammation, **P**= proliferation, **T**= trauma

1.1.3 Psoriasis, a disorder of cutaneous inflammation and epidermal proliferation

Psoriasis affects about 2% of the world's population and shows a polygenic pattern of inheritance. Oddly, the disease is precipitated by various non-genetic triggering factors, both internal and environmental, such as local trauma, stress, hormones, injections, drugs and hypocalcaemia. Sharply defined, thickened, red lesions may appear anywhere on the body, often beginning on the elbows or knees; these tend to grow slowly in size until much of the body surface is involved. Several less common variants exist, e.g. generalized pustular psoriasis, persistent palmoplantar pustulosis and acrodermatitis continua superativa; extracutaneous manifestations are found in the nails, the mucosal membranes and the joints.

At the microscopical level, psoriasis is characterized by acanthosis and parakeratosis, absence of the granular layer, thinning of the suprapapillary portion of the stratum Malpighii, increased cells and mitotic figures in the basal and suprabasal cell-layers, elongation of the rete ridges and long oedematous and often club-shaped papillae. In the papillary dermis there is tortuosity and dilatation of the capillaries and a mild mononuclear inflammatory infiltrate. Besides accumulation of polymorphonuclear leucocytes in the epidermis (micropustules and microabscesses) is a characteristic feature. Figure 3 shows the histology of the psoriatic lesion.

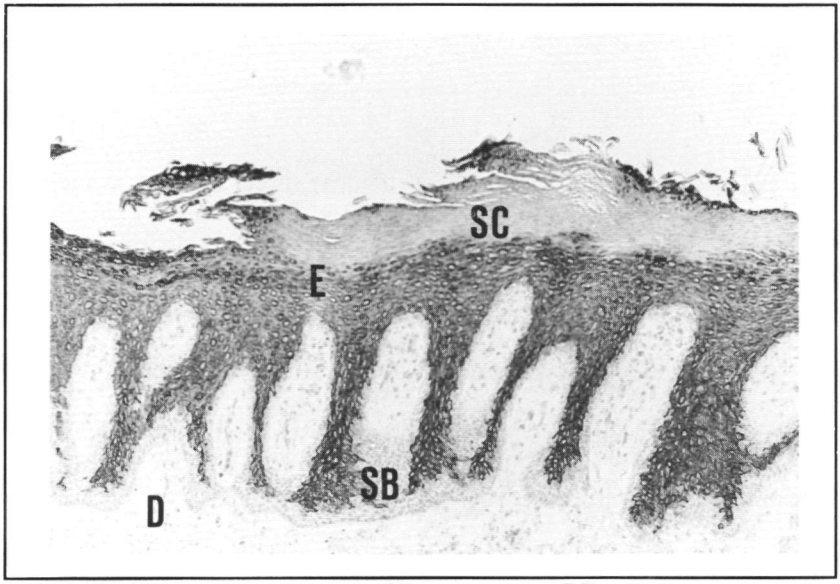

Fig.3 **D**= dermis, **E**= epidermis, **SB**= str.basale, **SC**= str. corneum

At a biochemical level, lesional skin also shows many differences compared to the distant, clinically uninvolved skin. In general, changes seen in the lesion are indistinguishable from the normal response of healthy skin to any form of injury. For example, epidermal growth factor binding,[4] phospholipase C activity,[5] protein kinase C activity,[6] arachidonic acid metabolites[7] and ornithine decarboxylase activity[8] are all increased in the psoriatic plaque. Some differences may exist between clinically uninvolved skin and the skin of healthy persons, without a family-history or signs of psoriasis. For example, increased activity of phospholipase A_2[9] and ornithine decarboxylase[10] has been claimed in psoriatics.

From a clinical and pharmacological point of view, every patient has his own psoriasis. A permanent cure for psoriasis is not yet possible. Therefore, a broad range of symptomatic 'classical' therapies is being used. Each one has its own specific limitations. Corticosteroids induce short remission periods and long-term treatment causes atrophy of the skin or even Cushing syndrome. Dithranol (anthralin) immobilizes the patient, since treatment necessitates several hours a day. Phototherapy with ultraviolet B radiation or photochemotherapy with ultraviolet A combined with psoralen-capsules (PUVA) both have cumulative carcinogenic potential. Finally, methotrexate and aromatic retinoids require a frequent and sustained supervision of the patient. Therefore, resolution of the pathogenesis will provide a more rational, effective therapy; a general statement in medicine.

1.1.4 The black-box-model

The biochemical analysis and identification of the enzymes, hormones, cytokines and structural proteins that are involved in epidermal inflammation, proliferation and differentiation, have provided the basis for our explanation of the normal physiology of the skin. But an understanding of how epidermal cells respond to their (pathologic) internal and external environments will depend on elucidating their interactions, i.e. the behavior of these biochemical agents in time. This will enable us to unravel the etiologic factors of the many still unsolved skin disorders; the fascinating challenge of dermatological research.

One possible way to investigate those variables is the use of the so called black-box model. Give a defined pulse to the epidermis, measure an output system (in time) and

argue what might have happened. This model can be easily put into practice in animals *in vivo*, especially rodents. This approach allows evaluation of the effects of substances such as new drugs and toxic or carcinogenic chemicals. The results of this approach have provided many beneficial new insights and therapies, but unfortunately it is not possible to extrapolate all these data to the human situation. Both biochemically and histologically human skin is not equivalent to rodent skin and many dermatological diseases that affect mankind are not represented in the animal world. Therefore, in this thesis we worked with a black-box model applicable to the human situation *in vivo*.

As defined tape stripping of the stratum corneum or irradiation with a single dose of ultraviolet B was used to cause a standardized, hyperproliferative response. Tape stripping comprises successive applications of Sellotape® to a sharply defined area until the surface becomes glistening; removal of the stratum corneum is then considered to be complete. This painless procedure causes micro-traumata in membranes, resulting, amongst other consequences, in the release of plasma and arachidonic acid metabolites. This finally leads to accumulation of polymorphonuclear leukocytes in the skin, an increased number of mitoses, hyperplasia and parakeratosis.[11,12] There is a close histological similarity between the minute papules that develop 6-96 hours after tape stripping and the spontaneous pre-pinpoint papules that can be found in the initial stage of the psoriatic lesion.

Irradiation with ultraviolet B is preceded by individual measurements of the minimal erythema dose (MED), to correct for skin type and epidermal thickness. During experiments 3x the MED is given, causing diffuse damage of epidermal structures,[13]

resulting, for example in the release of interleukin-1,[14] arachidonic acid metabolites and lysosomal hydrolases. Finally this also leads to the hyperproliferative response mentioned above, although - probably due to the delaying repair of DNA - in a later stage.[15]

As output system we used measurements of the activity of ornithine decarboxylase, an enzyme thought to be involved in proliferation and differentiation. Figure 4 shows a schematic overview of this black-box-model.

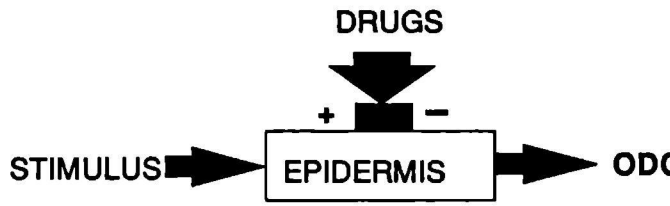

Fig.4

1.2 Ornithine decarboxylase (ODC)

1.2.1 Biochemistry and physiology of ODC

The first account indicating that putrescine is formed by the decarboxylation of ornithine in microorganisms dates back to 1940[16] and it took until 1968 before mammalian ODC was discovered[17]. Soon afterwards it became clear that its activity is always enhanced in response to anabolic stimuli[18], it possesses an extremely short molecular half-life of only 10-20 min[19], and pyridoxal phosphate is used as a cofactor[20].

In fact, the only important biochemical function of ODC is to catalyze the conversion of L-ornithine into putrescine and CO_2. A variety of mammalian ODC's is specific for the L-isomer of ornithine and does have K_M values of about 0.1 mM[21]. In vitro, mammalian ODC's are strongly dependent on the presence of reducing agents for maximal activity[22], but except for pyridoxal phosphate no other activators of the enzyme in normal tissues are known. Many extrahepatic cells (including keratinocytes), which lack the other enzymes of the urea cycle, contain arginase and are able to generate ornithine from arginine and use ornithine as a substrate for ODC[22,23]. In animals and humans, ornithine is also available from the plasma.

Providing putrescine for polyamine synthesis is the physiologic function of ODC. Putrescine in turn is indirectly converted into spermidine and spermine (the "genuine" poly-amines) by S-adenosyl-methionine-decarboxylase (SAM-DC). Cells cannot grow at their normal rate if either function is prevented, unless alternative pathways for polyamine synthesis are present (i.e. in mutant E. coli[24] or mammalian cells[25]) or exogeneous

polyamines are provided (i.e. by diet[26] or gut-flora[27]). Many negative and positive feed-back mechanisms do exist in these pathways[28] (see Fig. 5). Putrescine directly inhibits ODC and stimulates S-adenosyl-methionine decarboxylase (SAM-DC); at the ribosomal level spermidine blocks the translation of ODC mRNA and spermine blocks the translation of SAM-DCmRNA. Spermine and spermidine can be reconverted into putrescine by their respective N-acetyltransferase, however, putrescine cannot be reconverted into ornithine. Therefore, at least in cell types containing very low basal ODC activity (epidermis!), ODC can be considered as the rate-limiting step in polyamine biosynthesis.

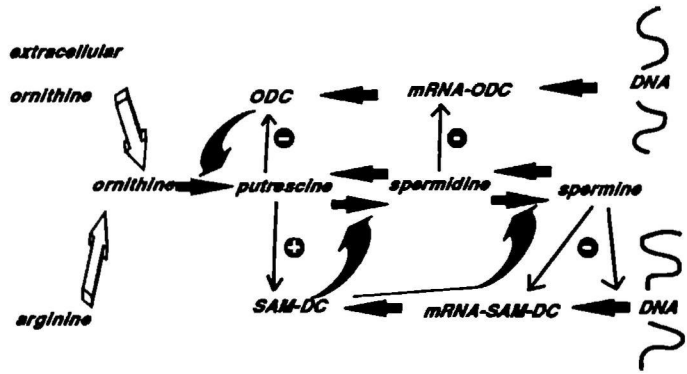

Fig.5

Under physiological conditions polyamines behave as organic cations and therefore are presumed to associate with anionic binding sites in e.g. nucleic acids and proteins through electrostatic interactions[29]. There are indications for specific binding of spermine with tRNA and DNA, which probably stabilizes the confirmation of the anticodon loop of

tRNA and induces bending and aggregation of DNA. Evidence also exists that the chromatin structure is stabilized by polyamines[30]. They are involved in the synthesis of macromolecules as well. Protein synthesis is clearly blocked by inhibition of polyamine synthesis, as shown by a decrease in the incorporation of radioactive amino acids both in vitro[31] and in vivo[32]. The mechanism by which polyamines are involved in DNA replication are as yet unclear. Perhaps replicon initiation rather than chain elongation is affected by polyamine deficiency[33]. During inhibition of ODC, the level of decarboxylated SAM becomes highly increased, which results in inhibition of SAM mediated DNA methylation[34]. This process may contribute to the antiproliferative effects of cellular polyamine depletion by inhibition of ODC.

Finally, of the many functions polyamines have on various processes in living cells, another important, although rather poorly documented one has to be mentioned. They can be covalently linked with glutamate residues of proteins by the enzyme transglutaminase[35,36]. This results in crosslinking of proteins, a specific differentiation process in the formation of the stratum corneum[37]. However, confusion remains in the early literature especially regarding the differences between intracellular / soluble and membrane-bound / "epidermal" transglutaminases. Besides, polyamine synthesis inhibitors induce terminal differentiation of certain tumors.[38]

In summary, from a biochemical point of view, we may conclude that ODC seems to be a valuable tool in studying the regulation processes that are involved in at least proliferation and maybe also differentiation. In the next paragraph its applications and present status in dermatological research are discussed.

1.2.2 Epidermal ODC[39,40,41]

ODC activity is normally very low or not measureable in undamaged, healthy skin of both rodents and humans. In rodents, ODC induction follows the application of certain tumor promoting phorbol esters (peak after 6-8 h) or irradiation with ultraviolet-B (peak after 24 h). In addition, hair plucking and tape stripping have been shown to induce ODC; the latter with a similar induction curve following application of tumor promoting agents (TPA). Increasing age has been reported to decrease the ability of inducing epidermal ODC, at least in rats. Except for the application of TPA, the same modes of induction have been studied in humans. However, in the case of UV-B irradiation only, a complete induction curve is available, comparable with rodents. No data are known regarding the effects of increasing age.

Several drugs that block ODC induction have been mentioned in the literature, revealing some insight into the biochemical pathways of ODC induction and hence epidermal (hyper)proliferation. In rodents corticosteroids, both cyclo-oxygenase- and (or) lipoxygenase-inhibitors, retinoic acid, vitamin D_3 and inhibitors of protein kinase C (PKC) have been shown to reduce ODC induction. In humans (at least *in vivo*) neither ligands of the steroid hormone receptor superfamily, eicosanoids nor PKC-inhibitors have been investigated regarding their influence on epidermal ODC induction.

In psoriatic patients, ODC activity is clearly elevated in the lesional plaque compared to the clinically uninvolved skin. This increased ODC activity proved to be reduced *in vivo* by corticosteroids and retinoic acid. A few reports only described slightly increased ODC activity in the uninvolved skin compared to the epidermis of healthy

controls and in a single report this difference became even larger following an induction stimulus.

The data mentioned in this chapter lead to an hypothetical explanation regarding the pathways of ODC induction in rodent epidermis. In an oversimplified way, this has been scheduled in Fig. 6.

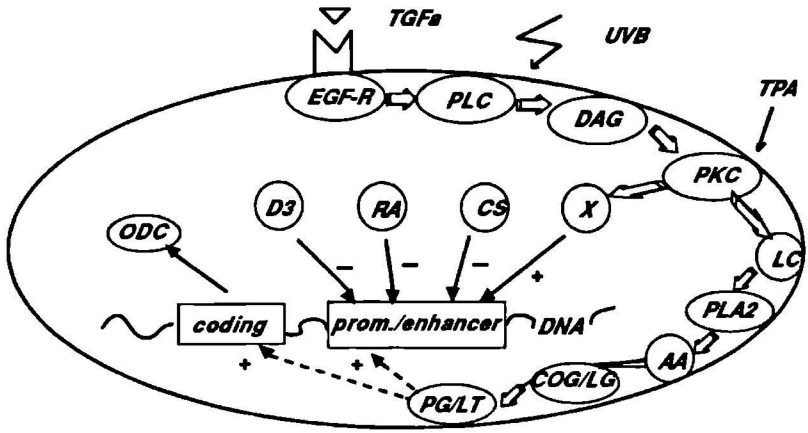

Fig. 6

Legend to Fig. 6 AA= arachidonic acid, CS= corticosteroid, COG= cyclooxygenase, D_3= vitamin D_3, DAG= diacylglycerol, EGF-R= epidermal growth factor receptor, LC= lipocortin, LG= lipoxygenase, LT= leucotrienes, PG= prostaglandins, PKC= protein kinase C, PLA_2= phospholipase A_2, PLC= phospholipase C, RA= retinoic acid, TGFα= transforming growth factor α, TPA= tetradecanoylphorbol-acetate, UVB= ultraviolet B, X= transcription factor(s)

1.3 Aim of the thesis

In paragraph 1.1 and 1.2 it has become clear that the investigation of induction pathways of ODC may lead to a better understanding of the (patho)physiology of proliferation and may give insight into therapeutic mechanisms of antipsoriatics. And especially in paragraph 1.2.2 it is obvious that many questions regarding epidermal ODC induction still have to be answered in humans, because data that are well established in rodent epidermis have never been checked in humans and also since some data are conflicting even for rodent epidermis. Concerning the latter, for example, inhibition of arachidonic acid release by corticosteroids surely blocks ODC induction, but whether its biochemical "end-products", prostaglandins and/or leucotrienes are responsable for this action, remained uncertain.

Therefore we started first investigating induction curves following tape stripping of both the epidermis of healthy controls and the uninvolved skin of psoriatic patients, in order to establish: a) are basal ODC activity levels measurable?, b) what is the time and level of the peak of maximal ODC activity following this induction technique? and c) do both basal and peak ODC activities differ between healthy subjects and psoriatic patients (indicating a key in the pathogenesis)? This will be described in chapter II; in addition a first attempt was made to investigate the influence of the arachidonic acid cascade on epidermal ODC induction by the use of a topical corticosteroid and a systemical cyclooxygenase inhibitor. In chapter III the effects of a topical cyclooxygenase- and lipoxygenase- inhibitor regarding epidermal ODC induction are reported.

Vitamin D_3 has been shown to block ODC induction in rodent epidermis only. Therefore it became interesting to find out whether this was also true in human epidermis and moreover if this would explain a / the mode of action of calcipotriol, the new anti-psoriatic vitamin D_3 analogue. Besides, since so many ligands of the so-called steroid hormone receptor superfamily (corticosteroids, retinoic acid, vitamin D_3) proved to be both inhibitors of ODC induction and potent antipsoriatic drugs, it seemed of interest to study the effects of another member, estriol. These questions are addressed in chapter IV.

Combining the data of chapter III and IV, raised the possibility that ODC could be used as a marker-enzyme to test (new) antipsoriatic therapies. In chapter V, therefore, the concept that inhibitors of epidermal ODC induction would be antiproliferative drugs was tested for a) sphingosine, a potent PKC-inhibitor, b) isoquinoline, possibly a PKC inhibitor or otherwise an active compound of antipsoriatic coal-tar, c) tannic acid, a substance that had proven empirically to inhibit ODC induction in rodent skin and d) cyclosporin A, a recently registered antipsoriatic drug with conflicting data regarding its influence on epidermal proliferation.

References

1. King LE, Gates RE, Stocheck CM et al. The EGF/TGFα receptor in skin. J Invest Dermatol 94: 164S-170S, 1990
2. Verma AK, Pong R-C, Erickson D. Involvement of protein kinase C activation in ornithine decarboxylase gene expression in primary culture of newborn mouse epidermal cells and in skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate. Cancer Res 46: 6149-6155, 1986
3. Flower RJ. The mediators of steroid action. Nature 320: 20, 1986

4. Nanney LB, Stocheck CM, Magid M et al. Altered ^{125}I epidermal growth factor binding and receptor distribution in psoriasis. J Invest Dermatol 86: 260-265, 1986

5. Bartel RJ, Marcelo CL, Voorhees JJ. Partial characterization of phospholipase C activity in normal, psoriatic uninvolved and lesional epidermis. J Invest Dermatol 88: 447-451, 1987

6. Horn F, Marks F, Fisher GJ et al. Decreased protein kinase C activity in psoriatic versus normal epidermis. J Invest Dermatol 88: 220-222, 1987

7. Ziboh VH, Tamara L, Casebolt BS et al. Biosynthesis of lipoxygenase products by enzyme preparations from normal and psoriatic skin. J Invest Dermatol 83: 426-430, 1984

8. Lowe NJ, Breeding J, Russel DH. Cutaneous polyamines in psoriasis. Br J Dermatol107: 21-26, 1982

9. Ilchyshyn A, Ilderton E, Kingsburry JA et al. Evidence that the raised level of phospholipase A_2 in the uninvolved epidermis of psoriasis is caused by phoshorylation of an inhibitor. Br J Dermatol 111: 721-722, 1984

10. Bouclier M, Elbaze P, Milano G et al. Hyper-induction of skin ornithine decarboxylase activity in psoriatic patients. Br J Dermatol 115: 193-198, 1986

11. van de Kerkhof PCM, van Rennes H, de Grood RM et al. Response of the clinically uninvolved skin of psoriatic patients to standardized injury. Br J Dermatol 109: 287-294, 1983

12. Chang A, de Jongh GJ, Mier PD, van de Kerkhof PCM. Enzymatic quantification of polymorphonuclear leucocytes in normal and psoriatic skin following standardized injury. Clin Exp Derm 13: 62-66, 1988

13. Gilchrest BA, Soter NA, Stoff JS et al. The human sunburn reaction: Histologic and biochemical studies. J Am Acad Dermatol 5: 411-422, 1981

14. Dinarello CA. Cytokines: Interleukin 1 and tumor necrosis factor. In:*Inflammation: Basic principles and clinical correlates* (chapter 12); JI Gallin, IM Goldstein, R Snyderm (ed.), Raven Press, NY, 1988;195-208

15. Epstein JH, Fukuyama K, Fye K. Effects of ultraviolet radiation on the mitotic cycle and DNA, RNA and protein synthesis in mammalian epidermis in vivo. Photochem Photobiol 12: 57-65, 1970

16. Gale EF. The production of amines by bacteria. Biochem J 34: 392, 1940

17. Jänne J, Raina A. Stimulation of spermidine synthesis in the regenerating rat liver: Relation to increased

ornithine decarboxylase activity. Acta Chem Scand 22: 1349, 1968

18. Jänne J, Pösö H, Raina A. Polyamines in rapid growth and cancer. Biochim Biophys Acta 473: 241, 1978

19. Russell DH, Snyder SH. Amine synthesis in regenerating rat liver: Extremely rapid turnover of ornithine decarboxylase. Mol Pharmacol 5: 253, 1969

20. Rahiala EL, Kekomäki M, Jänne J et al. Inhibition of pyridoxal enzymes by l-canaline. Biochim Biophys Acta 227: 337, 1971

21. Pegg AE, McGill S. Decarboxylation of ornithine and lysine in rat tissues. Biochim Biophys Acta 568: 416, 1979

22. Pegg AE, Williams-Ashman MG. Biosynthesis of putrescine, in *Polyamines in Biology and Medicine*, DR Morris, LJ Marton (eds). M Dekker, NY, 1981;3

23. Pegg AE, McCann PP. Polyamine metabolism and function. Am J Physiol 243: C212, 1982

24. Tabor CW, Tabor H. Polyamines in microorganisms. Microbiol Rev 49: 81, 1985

25. Pohjanpelto P, Hölttä E, Jänne OA. Mutant strain of Chinese hamster ovary cells with no detectable ornithine decarboxylase activity. Mol Cell Biol 5: 1385, 1985

26. Bardocz S, Brown DS, Grant G, Pusztai A. Luminal and basolateral polyamine uptake by rat small intestine stimulated to growth by Phaseolus vulgaris lectin phytohaemagglutin in vivo. Biochim Biophys Acta 1034: 46-52, 1990

27. Osborne DL, Seidel ER. Microflora-derived polyamines modulate obstruction-induced colonoc mucosal hypertrophy. Am J Physiol 256:G1049- G1058, 1989

28. Pegg AE. Recent advances in the biochemistry of polyamines in eukaryotes. Biochem J 234: 249-262, 1986

29. Marton LJ, Morris DR. Molecular and cellular functions of the polyamines. In: PP McCann, AE Pegg, A Sjoerdsma (eds.) *Inhibition of polyamine metabolism. Biological significance and basis for new therapies.* Academic Press, Orlando, 1987; 121-140

30. Snyder RD. Polyamine depletion is associated with altered chromatin structure in HeLa cells. Biochem J 260: 697-704, 1989

31. Igarashi K, Morris DR. Physiological effects in bovine lymphocytes of inhibiting polyamine synthesis with ethylglyoxal bis-guanylhydrazone. Cancer Res 44: 5332-5337, 1984

32. Seiler N, Sarhan S, Grauffel C et al. Endogeneous and exogeneous polyamines in support of tumor growth. Cancer Res 50: 5077-5083, 1990

33. Krokan H, Eriksen A. DNA synthesis in HeLa cells and isolated nuclei after treatment with an inhibitor of spermidine synthesis, methyl glyoxal bis-guanylhydrazone. Eur J Biochem 72: 501-508, 1977

34. Heby O, Persson L, Smith SS. Polyamines, DNA methylation and cell differentiation. In: V Zappia, AE Pegg (eds.): *Progress in polyamine research.* Plenum Press, NY, 1989; 291-299

35. Folk JE, Park MH, Chung SI et al. Polyamines as physiological substrates for transglutaminase. J Biol Chem 255: 3695-3700, 1980

36. Heby O. Role of polyamines in the control of cell proliferation and differentiation. Differentiation 19: 1-20, 1981

37. Rice RH, Green H. The cornified enveloppe of terminally differentiated human epidermal keratinocytes consists of cross-linked protein. Cell 11: 417-422, 1977

38. Kelly M, McCann PP, Schindler J. Alterations in polyamine metabolism during embryonal carcinoma cell differentiation in vitro. Dev Biol 111: 510, 1985

39. Boutwell RK, O'Brien TG, Verma AK et al. The induction of ornithine decarboxylase activity and its control in mouse skin epidermis. Adv Enzyme Regul 17: 89-112, 1978

40. Lowe NJ. Epidermal ornithine decarboxylase, polyamines, cell proliferation, and tumor promotion. Arch Dermatol 116: 822-825, 1980

41. Lowe NJ. Cutaneous polyamines and their biosynthetic enzymes. In: HH Roenigk, HI Maibach (eds.)*Psoriasis*, M Dekker, NY, 1985; 249-254

Chapter II

THE INDUCTION OF ODC FOLLOWING SELLOTAPE STRIPPING

2.0 INDUCTION OF ODC FOLLOWING SELLOTAPE STRIPPING IN NORMAL AND PSORIATIC SKIN

W.P. Arnold, C.A.E.M. van Hooijdonk, P.D. Mier, P.C.M. van de Kerkhof

This work was presented at the 20th annual meeting of the E.S.D.R., Turin, June 9th - 12th, 1990

Ornithine decarboxylase (ODC) was measured in the epidermis of healthy volunteers and the uninvolved skin of psoriatic patients at various times after sellotape stripping. Basal levels were less than 1 pmol/min/mg protein. Activity peaked to a maximum of 86 pmol/min/mg protein after 8 h; this was followed by an abrupt decline to a lower level which remained relatively constant for at least 36 h. No difference was seen between the response of controls and psoriatic patients. Pretreatment with topical corticosteroids reduced peak ODC levels to about one-half, but oral indomethacin had no effect.

INTRODUCTION

ODC is the rate-limiting enzyme for polyamine biosynthesis. Cellular ODC levels are linked to the phase of the cell cycle. Quiescent or early G_1 cells show low activity, but there is an abrupt increase in late G_1 which provides the polyamines that are required for DNA synthesis. Thus, ODC is a valuable 'marker enzyme' for cellular proliferation.[1,2]

Most of the published investigations regarding epidermis have used rodents, where ODC induction has been demonstrated following injury (e.g. tape stripping[3] or ultraviolet radiation[4-9]) or the application of tumour-promoting agents such as tetradecanoylphorbol acetate (TPA).[10-12] Several groups have reported increased ODC levels in the lesions of psoriasis, and shown that these normalize following therapy.[2] More recently it has been claimed that ODC is 'hyper-inducible' in the psoriatic patient.[13] However, to our knowledge the time-course of ODC induction following injury has never been investigated in human skin. Here we report ODC levels following tape stripping in healthy controls and psoriatic patients, and describe the effects of pretreatment with topical corticosteroids or oral indomethacin.

METHODS

Subjects

The control group consisted of 19 volunteers (10 male, nine female; age range 21-57 years), none of whom had a personal or family history of psoriasis. The psoriatic group comprised eight patients (five male,three female; age range 17-55 years) with chronic plaque psoriasis, who had received no therapy for at least 4 weeks prior to investigation. The use of aspirin or other NSAIDs was specifically excluded in all subjects during or immediately prior to experiments. Informed consent was obtained from all the subjects and the study had the approval of the Medical Ethics Committee.

Sellotape^{R} stripping was carried out as described previously[14], using a plastic

template to restrict the damage to sharply defined areas of 2 x 3 cm, and the stripped sites covered with a non-occlusive dressing. At appropriate times, epidermal slices of approximately 1 cm^2 were cut from the centre of each site using a Castroviejo keratotome (Storz Instr. Co.) set for a depth of 0.3 mm after cooling the skin with an ethylchloride spray. Biopsies were snap frozen in liquid nitrogen and kept at this temperature prior to analysis.

Six volunteers were pretreated with oral indomethacin, taking 75 mg 14 h and 2 h prior to tape stripping. A second group, consisting of five volunteers were pretreated with either topical betamethasone dipropionate or a base cream under occlusion for 14 h prior to stripping. The sites were thoroughly cleaned with 70 % alcohol immediately prior to stripping, and biopsies taken for ODC measurement after a further 8 h.

ODC measurement

This was a modification of a previously described method[15].Biopsies were homogenized in 0.5 ml of a freshly prepared buffer (50 mM Tris, 50 µM pyridoxal phosphate and 2 mM dithiothreitol at pH 7.3) using an ice-cooled Potter-type grinder. The reaction was initiated by adding 80 µl of homogenate to 20 µl of a solution containing 2 mM L-ornithine and 0.1 µCi L-[1-^{14}C]ornithine (New England Nuclear, Boston, MA, U.S.A.) in plastic tubes. After fitting each tube with a plastic cap carrying 5 cm^2 filter paper moistened with 20 µl 10 % KOH, the mixture was incubated at 37 °C for 45 min. The reaction was stopped by adding 100 µl 1M HCl, and incubated at 37°C for a further 30 min to ensure complete absorption of $^{14}CO_2$ on to the paper. Radioactivity was measured by scintillation counting

using an Isocap-300 (Searle Instruments B.V.); the paper was placed directly into the scintillation vial containing 4.5 ml scintillation fluid (Aqua Luma Plus) without prior dissolution. All samples were assayed in duplicate, and a complete reagent blank was included with each batch.

Preliminary experiments were carried out as described above to verify the linearity of the reaction with respect to time for incubation periods up to 60 min.

Protein measurement

An aliquot of the homogenate was diluted 1 : 5 with homogenizing buffer and centrifuged (10 min, 15,000 g). Protein was determined in the clear supernatant by direct fluorescence (λ_{EX}= 278 nm, λ_{EM}= 340 nm) using bovine serum albumin as standard. Linearity was confirmed in the range of 1-200 µg/ml protein.

Statistical methods

Analysis of variance was carried out for time and diagnosis using the SAS package and a VAX computer system.

RESULTS

The total reagent blank (including instrument background) averaged about 20 c.p.m. If an increment of 5 c.p.m. above this level is regarded as the minimum required to detect ODC

activity, and assuming a typical value of around 250 µg/ml for the concentration of soluble protein in the original homogenate, the absolute sensitivity of the method may be calculated to be about 1 pmol CO_2 released per min per mg protein. The assay was linear with respect to time for about 45 min, but showed a clear decline at 1 h. An incubation period of 45 min was therefore used for routine measurements. The reproducibility was good and duplicate assays averaged within ± 4.3 % of the mean.

No ODC activity could be detected in biopsies taken immediately after stripping (i.e. less than 1 pmol/min/mg protein). The time curve during the following 36 h is shown in Figure 1. An abrupt increase at 4 h reached a peak of 86 pmol/min/mg protein at 8 h. This was followed by an equally rapid diminution, reaching a level which remained relatively constant until at least 36 h post-stripping. The 24-h specimens, although averaging rather higher than the 16 h and 36 h values, did not differ significantly from the latter groups. No significant difference in ODC activity was observed between healthy volunteers and the clinically uninvolved skin of psoriatic patients at any of the times studied (4, 8 and 16 h).

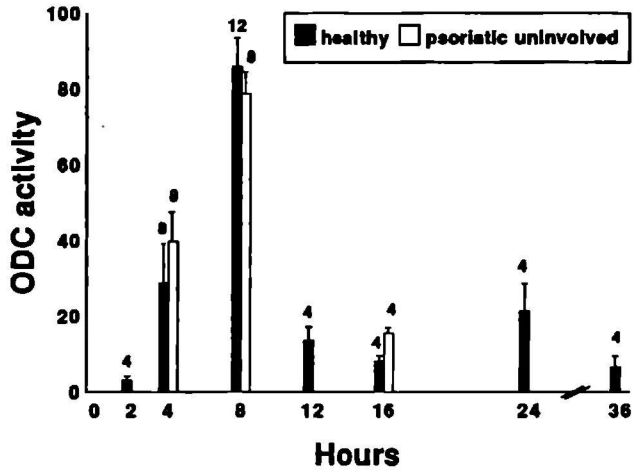

Fig. 1

ODC levels in healthy and psoriatic uninvolved epidermis at various times after tape stripping. All values are expressed as pmol/min/mg protein (mean ± SEM). Numbers of specimens are shown at the top of the bars.

The peak ODC levels in sites pretreated with betamethasone dipropionate averaged 45 pmol/min/mg protein, around one-half the value of either the untreated controls or the sites which had been occluded with base only (Table 1). By contrast, subjects receiving oral indomethacin prior to biopsy did not show any significant difference from the control group.

Table 1 Effects of pretreatment with indomethacin or betamethasone dipropionate on epidermal ODC measured 8 h after tape stripping. Values are expressed as pmol/min/mg protein (mean ± SEM). The control values for the indomethacin experiment are from Figure 1; for the betamethasone dipropionate experiment the control sites were pretreated with base cream (betametasone dipropionate vs. control, $P < 0.05$)

Treatment	Experimental group	Control group
Indomethacin	82 ± 8 (n = 6)	86 ± 8 (n = 12)
Betamethasone dipropionate	45 ± 7 (n = 5)	87 ± 5 (n = 5)

Table 1

DISCUSSION

The time-course of ODC induction in human subjects following stripping was similar to that following the application of tumor-promoting agents to mice, where enzyme activities peak around 6 - 10 h.[10-12] A similar value of 6 h has been reported following stripping the skin of the hairless rat,[3] but peak ODC levels were seen 14 - 28 h following treatment of mice with UVB.[4,5,8,9] Absolute values in rodent epidermis show wide variation, probably due to differences in methodology but most groups report basal ODC activity as being too low to measure.

Only one previous report has appeared regarding the effect of injury on ODC levels in human subjects.[13] These workers used an arbitrary induction period of 6 h, and compared the response of psoriatic patients to Sellotape^{R} stripping with that of healthy controls. Their findings differ in several respects both from previous work and to our

present observations. All specimens showed remarkably high basal levels, no significant difference being reported between psoriatic lesion and normal skin. At 6 h, ODC activity had doubled in healthy subjects, but in psoriatic patients had risen by a factor five- to ten fold. By contrast, our data (Fig.I) indicate at least an 80-fold stimulation which is identical in normal and psoriatic uninvolved skin. The reasons for these discrepancies are not clear. The only significant methodological difference is that the previous workers do not mention correcting their data for a reagent blank; this could account for the higher basal activity and hence the lower induction ratios.

There is considerable evidence that an arachidonic acid metabolite (presumably a cyclooxygenase product) is required for ODC induction. Thus the response to stimuli such as TPA applied to the skin of rodents is blocked by corticosteroids or cyclooxygenase inhibitors.[16-19] However, this data is difficult to extrapolate to human skin, especially using appropriate routes of administration and dosage schedules. We have shown in this study that betamethasone dipropionate results in a clear reduction of ODC levels following injury (Table I). Thus, in addition to their anti-inflammatory and other effects, corticosteroids may diminish proliferation via a reduction in polyamine availability.

The induction time following stripping was similar to that reported for TPA. Since the induction of ODC is mediated via activation of protein kinase C,[12] either directly (by TPA) or indirectly following injury, we conclude that the sequence of events between injury and protein kinase C activation is fairly rapid. Thus, the long delay between stripping and G_0 recruitment[20] occurs 'distal' to protein kinase activation, and cannot be explained by the slow release or diffusion of cytokines resulting from stripping. Also,

because the first wave of DNA synthesis begins around 32 h after stripping,[20] what is the function of polyamines peaking virtually a full cell cycle before this time? The reasons for this await further research.

REFERENCES

1 Lowe NJ. Epidermal ornithine decarboxylase, polyamines, cell proliferation and tumor promotion. Arch Dermatol 116: 822-825, 1980

2 Lowe NJ. Cutaneous polyamines and their biosynthetic enzymes. In: *Psoriasis* (HH Roenigk, HI Maibach, eds). New York: Dekker, 1985; 249-254

3 Bouclier M, Shroot B, Dionisius V et al. A rapid and simple test system for the evaluation of the inhibitory activity of topical retinoids on Sellotape stripping induced ODC activity. Dermatologica 169: 242, 1984

4 Lowe NJ, Verma AK, Boutwell RK. Ultraviolet light induces epidermal ornithine decarboxylase activity. J Invest Dermatol 71: 417-418, 1978

5 Verma AK, Lowe NJ, Boutwell RK. Induction of mouse epidermal ornithine decarboxylase activity and DNA synthesis by ultraviolet light. Cancer Res 39: 1035-1040, 1979

6 Lichti U, Patterson E, Yuspa SM. Retinoic acid differentially inhibits the induction of ODC by TPA and by germicidal ultraviolet light. Proc Am Assoc Cancer Res 20: 105, 1979

7 Lowe NJ, Breeding J. Antiinflammatory drug effects on ultraviolet light induced epidermal ornithine decarboxylase and DNA synthesis. J Invest Dermatol 74: 418-420, 1980

8 Lowe NJ, Breeding J. Retinoic acid modulation of ultraviolet induced epidermal ornithine decarboxylase activity. J Invest Dermatol 78: 121-124, 1982

9 Nemoto O, Koizumi H, Aoyagi T. A novel synthetic vitamin A-like compound (E-5166) inhibits UVB-stimulated epidermal ODC activity. Arch Dermatol Res 278: 407-409, 1986

10 Nakadate T, Yamamoto S, Aizu E, Kato R. Inhibition of TPA-induced tumor promotion and epidermal ODC activity in mouse skin by palmitoylcarnithine. Cancer Res 46: 1589-1593, 1986

11 Gupta AK, Fisher GJ, Elder JT et al. Sphingosine inhibits TPA-induced inflammation, ODC activity and activation of protein kinase C in mouse skin. J Invest Dermatol 91: 486-491, 1988

12 Yamamoto S, Kiyoto I, Aizu E et al. Differential inhibition by staurosporine, a potent protein kinase C inhibitor, of TPA caused skin tumor promotion, epidermal ODC induction, hyperplasia and inflammation. Carcinogenesis 10: 1315-1322, 1989

13 Bouclier M, Elbaze P, Milano G et al. Hyper-induction of skin ornithine decarboxylase activity in psoriatic patients. Br J Dermatol 115: 193-198, 1986

14 Van de Kerkhof PCM, van Rennes H, de Grood RM et al. Response of the clinically uninvolved skin of psoriatic patients to standardized injury. Br J Dermatol 109: 287-294, 1983

15 Hayashi SI, Kameji T. Ornithine decarboxylase (rat liver). Method Enzymol 94: 154-158, 1983

16 Verma AK, Ashendel CI, Boutwell RK. Inhibition by prostaglandin synthesis inhibitors of the induction of epidermal ODC activity, the accumulation of prostaglandins and tumor promotion caused by TPA. Cancer Res 40: 308-315, 1980

17 Nakadate T, Yamamoto S, Ishii M, Kato R. Inhibition of TPA-induced ep[idermal ODC activity by phospholipase A2 inhibitors and lipoxygenase inhibitor. Cancer Res 42: 2841-2845, 1982

18 Nakadate T, Aizu E, Yamamoto S et al. Inhibition of teleocidin-caused epidermal ODC induction by phospholipase A2-, cyclooxygenase- and lipoxygenase-inhibitors. Japan J Pharmacol 37: 253-258, 1985

19 Uzumaki H, Yamamoto S, Kato R. Effects of lipoxygenase- and cyclooxygenase inhibitors on the induction of ODC by TPA and isoprotenerol in mouse tissues in vivo. Carcinogenesis 7: 289-294,1986

20 Bauer FW, Boezeman JBM. Flow cytometric methods in human skin with respect to cell cycle kinetics. In: *Psoriasis: Cell Proliferation* (NA Wright, RS Complejohn, eds). Edinburgh: Churchill Livingstone 1983; 104-105

Chapter III

INFLUENCE OF EICOSANOIDS ON EPIDERMAL ODC INDUCTION

3.1 CYCLOOXYGENASE PRODUCTS DO NOT PARTICIPATE IN THE INDUCTION OF ODC IN HUMAN EPIDERMIS

W.P. Arnold, A.M.E. Lintsen, P.C.M. van de Kerkhof, P.D. Mier

This work was presented at the 21st annual meeting of the E.S.D.R., Copenhagen, June 1st - 4th, 1991

Extensive animal data have suggested that prostaglandins are involved in the epidermal induction of ornithine decarboxylase (ODC). The aim of the present study was to investigate their role during the induction of hyperproliferation in human skin.

Two groups of experiments were carried out. In the first topical indomethacin (Elmetacin®) or vehicle only were applied under occlusion on the backs of healthy volunteers, followed by sellotape stripping after 1 h; biopsies for ODC measurement were taken after a following 8 h. No difference was found between indomethacin versus control. In the second experiment test sites were irradiated with 3x the Minimal Erythema Dose of UV-B, immediately followed by application of indomethacin or vehicle only on the UV-irradiated sites. After 8 h biopsies were taken. The degree of erythema was markedly reduced in all the indomethacin treated sites, compared to the control, confirming that dosage and penetration were adequate. Nevertheless, ODC levels were again similar on both sites. These data indicate that cyclooxygenase products in human epidermis do not contribute to the induction of ODC.

INTRODUCTION

Ornithine decarboxylase (ODC) is the rate-limiting enzyme in the biosynthesis of polyamines, that are required for DNA synthesis. Therefore elevation of ODC activity is seen in various hyperproliferative situations. Basal ODC-levels are not detectable in normal epidermis, but induction of this enzyme has been demonstrated following injury (e.g. sellotape stripping,[1,2] UV-irradiation[3,4]) or application of tumour promoting agents.[5,6]

Extensive animal data have suggested that eicosanoids are essential for the epidermal induction of ODC.[5-14] In vivo studies in rodents report that the ODC induction is markedly suppressed by cyclooxygenase inhibitors,[5-9] by lipoxygenase inhibitors[10-12] or both.[13,14] Thus it remains uncertain whether prostaglandins, leukotrienes or both contribute to the epidermal ODC induction. These contradictory facts raise the question of what pathway is responsible in human skin.

The purpose of this study was to clarify whether prostaglandins are involved in the epidermal induction of ODC. We describe the effects of the topical cyclooxygenase inhibitor Elmetacin® (indomethacin 1%) on trauma- and UV-B induced ODC in human epidermis.

METHODS

Sellotape stripping was used as an inflammatory insult because data regarding the time-course and enzyme peak activities are well documented.[2] To ensure that the indomethacin reservoir in the stratum corneum remained available during the induction period we designed an additional experiment using UV-B. Epidermal ODC-activity was determined at its maximal level, 8 h after stripping.[2] By contrast maximum ODC-levels following UV-B irradiation are not achieved until 24 h,[15] which we confirmed in preliminary investigations (data not shown). Restricted by the pharmacokinetics of indomethacin[16] and for practical reasons, we nevertheless took skin samples 8 h after irradiation, when the ODC-activity was sufficient to permit accurate quantification.

Stripping experiment

This consisted of 9 healthy volunteers (4 male, 5 female, age range 18-58 years), without known hypersensitivity to Non-Steroidal Anti-Inflammatory Drugs (NSAIDs). The use of aspirin or other NSAIDs was not allowed during or immediately prior to the experiment. Indomethacin 1% (40 µl/cm^2) in an isopropanol solution, (Elmetacin^R, Luitpold Werk, München, BRD) or vehicle only, were applied within a plastic cylinder (1.75 cm^2) on two sites of the backs, evaporated with air under pressure and occluded with a non-toxic plastic foil. After 1 h sellotape stripping was carried out on both sites as described previously,[2] using a plastic template to restrict the damage to the pretreated areas. These were covered with a non-occlusive dressing. After a further 8 h biopsies (0.5 cm^2, 0.3

mm) were taken from the centre of each site using a Castroviejo Keratotome (Storz Instrument Co.) after cooling the skin with an ethylchloride spray. Biopsies were washed in cold phosphate buffered saline. After drying between filterpaper and weighing, they were snapfrozen in liquid nitrogen and kept at -20°C prior to analysis.

UV-B experiment

This again consisted of 9 healthy volunteers (4 male, 5 female age range 18-28 year), subject to the previous restrictions. The minimal erythema dose (MED) of every individual was measured, using a high-pressure Hg source (Solamed^{R}) at a distance of 20 cm. Three sites of their backs (2 cm^2) were irradiated with a dose of 3x the MED. Immediately after irradiation, application of indomethacin or vehicle only was carried out. In order to exclude any effect of the vehiculum in itself, biopsies were also taken of irradiated sites without treatment. The test-sites were left under occlusion for 8 h and biopsies were taken for ODC measurement as before.

ODC-measurement

This was a modification of a previously described method.[17] Biopsies were homogenized in 0.5 ml of a freshly prepared buffer (50 mM Tris, 50 µM pyridoxal phosphate and 2 mM dithiothreitol at pH 7.3) using an ice-cooled Potter-type grinder. The reaction was initiated by adding 80 µl of homogenate to 20 µl of a solution containing 2 mM L-ornithine and 0.1 µl L-[1-^{14}C] ornithine (0.1 mCi/ml, specific activity 52.3 mCi/mmol; New England Nuclear, Boston, MA, USA) in plastic tubes. After fitting each tube with a plastic cap

carrying 5 cm^2 filter paper moistened with 20 µl 10% KOH, the mixture was incubated at 37°C for 45 min. The reaction was stopped by adding 100 µl 1 M HCl, and incubated at 37°C for a further 30 min to ensure complete absorption of $14CO_2$ onto the paper. The paper was placed directly into a scintillation vial containing 4.0 ml scintillation fluid (Aqua-luma Plus) without prior dissolution and radioactivity was measured by scintillation counting using a Pharmacia Wallac 1410. All samples were assayed in duplicate, and a complete reagent blank was included with each batch.

Protein-measurements

An aliquot of the homogenate was diluted 1:5.5 with homogenizing buffer and centrifuged (10 min, 12.000 rpm). Protein was determined in the clear supernatant by direct fluorescence (λ_{EX}=278 nm, λ_{EM}=340 nm) using bovine serum albumin as standard.

RESULTS

The total reagent blank (including instrument background) averaged about 22 dpm. Duplicate assays averaged within $\pm$ 2.4% of the mean. In preliminary experiments dosage and duration of indomethacin application were investigated; doubling of both variables did not alter the ODC-levels.

In the first experiment, ODC activity was measured following sellotape stripping. Pre-treatment of the sites with indomethacin resulted in ODC levels averaging 160 $\pm$ S.E. 30 pmol/min/mg protein; this was not significant different from those pre-treated with

vehicle only (129 ± 30 pmol/min/mg protein; $P > 0.05$). Expressed on a weight basis activities were 2.9 ± S.E. 0.6 and 2.6 ± 0.6 pmol/min/mg respectively, again similar ($P > 0.05$).

The second experiments, using UV-B irradiation to stimulate ODC induction, were done to rule out the possibility that an intact stratum corneum might be required to provide a "reservoir" of indomethacin during the induction period. On a protein basis, ODC levels for UV + indomethacin, UV + vehicle and UV only were 13 ± S.E. 2.2, 14 ± 1.5 and 16 ± 2.3 pmol/min/mg protein respectively; on a weight basis the values were 0.21 ± S.E. 0.05, 0.24 ± 0.05 and 0.26 ± 0.06 respectively. The effects of indomethacin were not statistically significant using either reference variable ($P > 0.05$) in both cases). Remarkably, however, indomethacin application resulted in a clear reduction of erythema in all 9 subjects compared with UV + vehicle or UV only.

DISCUSSION

The capacity of indomethacin to block prostaglandin biosynthesis is well documented.[18] It has become clear from our results that prostaglandins do not contribute to the in vivo induction of ODC in human skin. This is especially clear after UV-B irradiation, where the test-sites treated with indomethacin showed a marked reduction of erythema, which established beyond doubt that dosage and penetration of the cyclooxygenase inhibitor were adequate.

Investigations in animals using UV irradiation showed that the release of

cyclooxygenase products was dependent upon both dose and wavelength,[19-21] observed prostaglandin concentration paralleled the intensity of erythema. Reduction of erythema and oedema after topical application of indomethacin was also reported. It has been suggested that leukotrienes, act initially direct on epidermal cells, for example by stimulating the transcription of interleukin-1 within 15 min of cell activation.[22] On the other hand cyclooxygenase metabolites act mainly indirectly via other cell types, such as capillary endothelial cells.[18] The epidermal injury-induced prostaglandins cause vasodilatation, followed by infiltration of inflammatory cells, such as polymorphonuclear leucocytes, in a later stage. The release of their "aggressive" mediators would then continue the inflammatory, hyperproliferative response, including induction of ODC. Thus the indirect inhibition of ODC would only become measurable after at least 24 h of continued prostaglandin inhibition by indomethacin.

Further investigation are in progress to test the validity of this concept.

REFERENCES

1. Bouclier M, Shroot B, Dionisius V, et al. A rapid and simple test system for the evaluation of the inhibitory activity of topical retinoids on sellotape stripping induced ODC activity. Dermatologica 1984; 169: 242.
2. Arnold WP, van Hooijdonk CAEM, Mier PD, et al. Induction of ornithine decarboxylase following sellotape stripping in normal and psoriatic skin. Br J Dermatol 1990; 123: 581-85.
3. Lowe NJ, Verma AK, Boutwell RK. Ultraviolet light induces epidermal ornithine decarboxylase activity. J Invest Dermatol 1978; 71: 417-18.

4. Verma AK, Lowe NJ, Boutwell RK. Induction of mouse epidermal ornithine decarboxylase activity and DNA synthesis by ultraviolet light. Cancer Res 1979; 39: 1035-40.

5. Verma AK, Rice HM, Boutwell RK. Prostaglandins and skin tumor promotion: inhibition of tumor promotor- induced ornithine decarboxylase activity in epidermis by inhibitors of prostaglandin synthesis. Biochem Biophys Res Comm 1977; 79: 1160-66.

6. Verma AK, Ashendel CL, Boutwell RK. Inhibition by prostaglandin synthesis inhibitors of the induction of epidermal ODC activity, the accumulation of prostaglandins and tumor promotion caused by TPA. Cancer Res 1980; 40: 308-15.

7. Lowe NJ, Breeding J. Antiinflammatory drug effects on UV light induced epidermal ODC and DNA synthesis. J Invest Dermatol 1980; 74: 418-20.

8. Lowe NJ, Connor MJ, Breeding J, et al. Inhibition of UV-B epidermal ODC induction and skin carcinogenesis in hairless mice by topical indomethacin and triamcinolone acetonide. Cancer Res 1982; 42: 3941-43.

9. Prunieras M, Delescluse C. Epidermal cell culture systems in skin pharmacology. Br J Dermatol 1984; 111S27:43-57.

10. Kato R, Nakadate T, Yamamoto S, et al. Inhibition of TPA induced tumor promotion and ODC activity by quercetin:possible involvement of lipoxygenase inhibition. Carcinogenesis 1983; 4: 1301-05.

11. Aizu E, Nakadata T, Jamamoto S, et al. Inhibition of TPA mediated epidermal ODC induction and skin tumor promotion by new lipoxygenase inhibitors lacking PK-C inhibitory effects. Carcinogenesis 1986; 7: 1809-12.

12. Nakadate T, Jamamoto S, Aizu E, et al. Inhibition by lipoxygenase inhibitors of 7-bromomethylbenz[α]anthracene-caused epidermal ODC induction and skin tumor promotion in mice. Carcinogenesis 1989; 10: 2053-57.

13. Nakadate T, Jamamoto S, Ishiëe M, et al. Inhibition of TPA caused epidermal ODC activity by PL-A2 inhibitors and lipoxygenase inhibitors. Cancer Res 1982; 42: 2841-45.

14. Nakadate T, Aizu E, Jamamoto, et al. Inhibition of teleocidin-caused epidermal ODC induction by PL-A2-, cyclooxygenase- and lipoxygenase inhibitors. Japan J Pharmacol 1985; 37: 253-58.

15. Rosen CF, Gajic D, Drucker DJ. Ultraviolet radiation induction of ODC in rat keratinocytes. Cancer Res 1990; 50: 2631-35.

16. Zacher J, Dittrich P, Wessinghage D, et al. Indomethacin concentrations in blood, synovia, synovial membrane and skin after repeated cutaneous application of alcoholic indomethacin solution. Akt Rheumatol 1986; 11: 104-107.

17. Hayaski SI, Kameji T. Ornithine decarboxylase (rat liver). Method Enzymol 1983; 94: 154-58.

18. Goldstein IM. Agents that interfere with arachidonic acid metabolism. In: Inflammation: Basic principles and clinical correlates (Gallin JI, Goldstein IM, Snijderman R, eds.). New York: Raven Press 1988: 935-945.

19. Imokawa G, Tejima T. A possible role of prostaglandins in PUVA-induced inflammation: implication by organ cultured skin. J Invest Dermatol 1989; 92:296-300.

20. Kligman LH, Kaidley KH. Wavelength dependence for ornithine decarboxylase induction in vivo. Photochem Photobiol 1986; 43: 649-54.

21. Young AR, Connor MJ, Lowe NJ. UV wavelength dependence for the induction of ornithine decarboxylase activity in hairless mouse epidermis. Carcinogenesis 1986; 7: 601-4.

22. Dinarello CA. Cytokines: interleukin 1 and tumor necrosis factor (cachectin). In: Inflammation: Basic principles and clinical correlates (Gallin JI, Goldstein IM, Snijderman R, eds.). New York: Raven Press 1988; 195-208.

3.2 THE INDUCTION OF ODC IN HUMAN EPIDERMIS IS INDEPENDENT OF LIPOXYGENASE AND CYCLOOXYGENASE PATHWAYS

W.P. Arnold, B.J.H. Pennings, P.C.M. van de Kerkhof

In vivo studies in rodents suggest that prostaglandins and/or leukotrienes are involved in the epidermal induction of ornithine decarboxylase (ODC). Recently, we have shown that, in human epidermis, prostaglandins are not involved in this process. Here we report the role of leukotrienes in epidermal ODC induction in human skin. Topical flufenamic acid (Dignodolin^{R}), vehicle, or nothing was applied under plastic occlusion to three sites on the backs of healthy volunteers. This was followed 1 h later by Sellotape^{R} stripping. After renewed application and occlusion for 8 h, biopsies were carried out for the estimation of ODC levels. There were no significant differences in the levels of ODC between the flufenamic acid treated and control sites. To confirm this finding, test sites were irradiated with 3 MED of UVB. This was immediately followed by the application of flufenamic acid, vehicle, or nothing to the three irradiated sites. After 8 h, biopsies were taken, and the levels of ODC were again similar in the flufenamic acid- and the vehicle-tested sites. The data indicate that, following Sellotape^{R} stripping or UVB irradiation, neither lipoxygenase nor cyclooxygenase products contribute to the *in vivo* induction of ODC in human epidermis.

INTRODUCTION

Ornithine decarboxylase (ODC) is the rate-limiting enzyme for the biosynthesis of polyamines. Because polyamines are required for the DNA synthesis, ODC is a valuable 'marker enzyme' for cellular proliferation. In normal epidermis, ODC is not detectable, but, in various hyperproliferative situations, an elevation of ODC activity is seen. The enzyme can be induced by injuries such as Sellotape[R] stripping[1,2] and UV irradiation,[3,4] or by application of tumor-promoting agents such as tetradecanoyl-phorbol-acetate (TPA).[5,6]

In vivo studies in rodents indicated that ODC induction was suppressed by cyclooxygenase inhibitors,[5-9] lipoxygenase inhibitors[10-12] or both.[13,14] Therefore, it has been suggested that prostaglandins, leukotrienes, or both are essential for epidermal ODC induction.[5-14] Recently we have shown that, in human epidermis, prostaglandins do not participate in the induction of ODC.[15] Here we address the role of leukotrienes in this process.

This study was carried out to determine whether leukotrienes are essential to the induction of ODC in human epidermis by examining the effect of the topical lipoxygenase- and cyclooxygenase inhibitor flufenamic acid (Dignodolin[R]) on both trauma- and UVB-induced ODC in human epidermis. Flufenamic acid penetrates the epidermis without degradation[16] and a 3% concentration is sufficient for suppression of lipoxygenase and cyclooxygenase in human skin.[17-19] No data are available regarding the effect of the agent on protein kinase C.

METHODS

Sellotape[R] stripping was used as an *in vivo* model for epidermal proliferation, because the data concerning the time course and enzyme peak activities are well documented.[2] To ensure that the flufenamic acid reservoir in the stratum corneum remained available during the induction period, an additional experiment was performed using UVB. The maximum epidermal ODC activity is measured 8 h after stripping[2] and 24 h after UVB irradiation.[20] For practical reasons, including the pharmacokinetics of flufenamic acid, we nevertheless took skin samples 8 h after irradiation, as the level of ODC activity was then sufficient for accurate quantification.

Stripping experiment

This experiment involved 8 healthy volunteers (5 male, 3 female, age range, 20-26 years). None were allowed to use none-steroidal anti-inflammatory drugs after, during, or immediately prior to the experiment. About 200 mg of Dignodolin[R] cream containing 3% flufenamic acid (Luitpold Werk, München, Germany) and the same amount of vehicle were each applied on a 3 cm^2 site on the back and then occluded under non-toxic plastic sheets. After 1 h, Sellotape[R] stripping[2] was carried out at these two sites and at an untreated control site. Again, flufenamic acid or vehicle were each applied on the pretreated sites. The three sites were then covered with a non-toxic plastic occlusion. After a further 8 h, biopsies (1 cm^2 x 0.2 mm.) were taken from the centre of each site using a Castroviejo keratome under local anaesthesia produced by cooling the skin with ethyl

chloride. The biopsies were washed briefly in cold phosphate-buffered saline, snap frozen in liquid nitrogen, and kept at -20°C prior to analysis.

UVB experiment

This experiment also involved 6 healthy volunteers (3 male, 3 female, age range 20-27 years). The minimal erythema dose (MED) for each individual was determined and three sites on their backs (3 cm^2) were each irradiated with 3 MED. Within one minute after irradiation, flufenamic acid, the vehicle only, or nothing was applied to these sites, which were left under plastic occlusion for 8 h. Keratome biopsies (1 cm^2 x 0.2 mm) were taken to determine the ODC levels.

Measurement of ODC

This was a modification of a previously described method.[21] The biopsies were homogenized in 0.5 ml Tris buffer at pH 7.3 using an ice-cooled Potter-type grinder. The reaction was initiated by adding 80 µl of homogenate to 20 µl of a solution containing 0.5mM L-ornithine and 0.1 µCi L-[1-^{14}C]-ornithine (specific activity 52.3 mCi/mmol; New England Nuclear, Boston, MA, U.S.A.) in plastic tubes. After fitting each tube with a plastic cap with 5 cm^2 filter paper moistened with 20 µl 10% KOH, the mixture was incubated at 37°C for 45 min. The reaction was stopped by adding 100 µl 1M HCl, and incubated at 37°C for a further 30 min to ensure complete absorption of $^{14}CO_2$ onto the paper. The paper was then placed into a scintillation vial containing 4 ml scintillation fluid without prior dissolution, and the radioactivity measured. All samples were assayed in

duplicate and a complete reagent blank was also included.

Protein measurements

An aliquot of the homogenate was diluted with the buffer and centrifuged. The protein content was determined in the clear supernatant by direct fluorescence (λ EX=278 nm, λ EM=340 nm), using bovine serum albumin as the standard.

RESULTS

The level of ODC following Sellotape^{R} stripping in the sites treated with flufenamic acid averaged 89 ± SE 8 pmol/min/mg protein. This was not significantly different from the areas treated with vehicle only, which averaged 100 ± SE 16 pmol/min/mg protein, or from the untreated Sellotape-stripped sites, which averaged 110 ± SE 21 pmol/min/mg ($P>0.05$ in both cases, according to the Wilcoxon ranking test).

UVB irradiation was used to stimulate the induction of ODC; this experiment was performed to rule out the possibility that an intact stratum corneum might be required to provide a reservoir of flufenamic acid during the induction period. The average level of ODC following UVB-irradiation and flufenamic acid treatment was 13.6 ± SE 1.3 pmol/min/mg protein. This was again not significantly different from the areas treated with UVB plus vehicle, which was 12.8 ± SE 3.1 pmol/min/mg protein ($P>0.05$). However, application of flufenamic acid resulted in a clear reduction of erythema in all six subjects compared with UVB plus vehicle or UVB only. There is a significant difference between

the average ODC levels of the cream-treated sites and the untreated UVB-irradiated sites, which was 22.9 ± SE 3.4 pmol/min/mg protein (Wilcoxon : p=0.025).

All these data are summarized in table I.

Table I	Treatment		
Induction	Nothing	Vehicle only	Fluf.ac.3 %
Tape stripping	110 ± 21	100 ± 16	89 ± 8
UVB irradiation	22.9 ± 3.4	12.8 ± 3.1	13.6 ± 1.3

Legend to table I

Mean ODC activities, expressed in pmol/min/mg protein (± SEM), 8 h after tape stripping or UV-B irradiation of none-, vehicle- or flufenamic acid-treated human epidermis.

DISCUSSION

During the last few years it has become clear that the response of the skin to Sellotape[R] stripping,[1,2] UVB irradiation,[3,4] experimental application of TPA[5,6] and the clinical manifestations of chronic inflammatory diseases such as psoriasis,[22] are all mediated by activation of the enzyme PKC. This enzyme has many functions, at least two of which are well-documented. First, it is responsible for initiation of the arachidonic acid (AA) cascade (at least in part by phosphorylation of the PLA_2 inhibiting lipocortin)[23] and thus the synthesis of prostaglandins and leukotrienes. Secondly, it activates the transcription factor

AP1, inducing the synthesis of many of the proteins required for cell division, such as ODC.[24] This relatively simple model has been made more complicated by the reports that AA products are essential for ODC activation, suggesting that there is cross-talk between these pathways.

We have previous established that, at least in human skin, prostaglandins are not required for the activation of ODC.[15] Since the present data make it clear that lipoxygenase products play no role in ODC induction either, we have reached the conclusion that (at least in the human) this cross-talk does not occur. Two possible limitations must be considered. Firstly, the stripping procedure removes the stratum corneum which acts as a 'reservoir' for topical drugs. This may be disregarded since the experiment with UVB irradiation also gave negative results. The clear reduction of erythema on the UVB-irradiated and flufenamic acid treated sites, also reported in animal studies,[18] confirms bio-availability of the flufenamic acid and demonstrates that AA metabolism is indeed suppressed. Secondly, the reduction of ODC levels following UVB irradiation and application of vehicle with flufenamic acid or vehicle alone, compared with untreated UVB irradiated sites, is of interest. A speculative explanation would be that application of any cream or ointment onto the stratum corneum improves the bio-availability of free sphingosine in human epidermis.[25]

The apparent discrepancy between human epidermis and that of rodents in the requirements for the activation of proteins such as ODC is surprising, since, in general, signal transduction mechanisms such as this are highly conserved throughout evolution. Comparative studies using parallel techniques are clearly indicated.

REFERENCES

1. Bouclier M, Schroot B, Dionisius V et al.: A rapid and simple test system for the evaluation of the inhibitory activity of topical retinoids on sellotape stripping induced ODC activity, *Dermatologica*, **169**: 242, 1984.

2. Arnold WP, van Hooijdonk CAEM, Mier PD, Van de Kerkhof PCM: Induction of ornithine decarboxylase following sellotape stripping in normal and psoriatic skiin, *Br J Dermatol*, **123**: 581-585, 1990.

3. Lowe NJ, Verma AK, Boutwell RK: Ultraviolet light induces epidermal ornithine decarboxylase activity, *J Invest Dermatol*, **71**: 417-418, 1978.

4. Verma AK, Rice HM, Boutwell RK: Induction of mouse epidermal ornithine decarboxylase activity and DNA synthesis by ultraviolet light, *Cancer Res*, **39**: 1035-1040, 1979.

5. Verma AK, Rice HM, Boutwell RK: Prostaglandis and skin tumor promotion: Inhibition of tumor promotor-induced ornithine decarboxylase activity in epidermis by inhibitors of prostaglandin synthesis, *Biochem Biophys Res Comm*, **79**: 1160-1166, 1977.

6. Verma AK, Ashendel CL, Boutwell RK: Inhibition by prostaglandin synthesis inhibitors of the induction of epidermal ODC activity, the accumulation of prostaglandins and tumor promotion caused by o-tetradecanoylphorbol-13-acetate, *Cancer Res*, **40**: 308-315, 1980.

7. Lowe NJ, Breeding J: Antiflammatory drug effects on UV light induced epidermal ODC and DNA synthesis, *J Invest Dermatol*, **74**: 418-420, 1980.

8. Lowe NJ, Connor MJ, Breeding J, Chalet M: Inhibition of UV-B epidermal ODC induction and skin carcinogenesis in hairless mice by topical indomethacin and triamcinolone acetonide, *Cancer Res*, **42**: 3941-3943, 1982.

9. Prunieras M, Delescluse C: Epidermal cell culture systems in skin pharmacology, *Br J Dermatol*, **111** (suppl. 27): 43-57, 1984.

10. Kato R, Nakadate T, Yamamoto S, Sugimura T: Inhibition of TPA induced tumor promotion and ODC

activity by quercetin: Possible involvement of lipoxygenase inhibition, *Carcinogenesis*, 4: 1301-1305, 1983.

11. Aizu E, Nakadate T, Yamamoto S, Kato R: Inhibition of TPA mediated epidermal ODC induction and skin tumor promotion by new lipoxygenase inhibitors lacking protein kinase C inhibitory effects, *Carcinogenesis*, 7: 1809-1812, 1986.

12. Nakadate T, Yamamoto S, Aizu E, Kato R: Inhibition by lipoxygenase inhibitors of 7-bromomethylbenz[α]anthracene-caused epidermal ODC induction and skin tumor promotion in mice, *Carcinogenesis*, 10: 2053-2057, 1989.

13. Nakadate T, Yamamoto S, Ishii M, Kato R: Inhibition of TPA induced epidermal ODC activity by PL-A_2 inhibitors and lipoxygenase inhibitors, *Cancer Res*, 42: 2841-2845, 1982.

14. Nakadate T, Aizu E, Yamamoto S et al.: Inhibition of teleocidin-caused epidermal ODC induction by PL-A_2-, cyclooxygenase- and lipoxygenase inhibitors, *Japan J Pharmacol*, 37: 253-258, 1985.

15. Arnold WP, Lintsen AME, Van de Kerkhof PCM, Mier PD: Cyclooxygenase products do not participate in the induction of ornithine decarboxylase in human epidermis, *Br J Dermatol*, 125: 577-579, 1991.

16. Norwach H, Eckenberger HP: Detection of flufenamic acid in the interior of the knee joint after cutaneous application, *Arzneim Forsch/Drug Res*, 36: 10, 1986.

17. Fruchtmann R, Krupla U, Pelster B: Hemmung des Lipoxygenase- und cyclooxygenaseweges im Arachdonsäure Metabolismus durch Etofenamat, *Akt Rheumatol*, 9: 165, 1984.

18. Winder CV, Wax J, Serrano B, Jones EM, McPhee ML: Anti-inflammatory and antipyretic properties of N-(α,α,α-trifluoro-m-tolyl)-anthranilic acid (Cl-440; flufenamic acid), *Arthritis Rheumat*, 6: 36, 1963.

19. Jacobi H, Breier P, Dell HD, Lorenz D: Pharmacology and toxicology of etofenamate, Artzneimittelforschung, 27: 1326-1333, 1977.

20. Rosen CF, Gajic D, Drucker DJ: Ultraviolet radiation induction of ornithine decarboxylase in rat keratinocytes, *Cancer Res*, 50: 2631-2635, 1990.

21. Hayaski SI, Kameji T: Ornithine decarboxylase (rat liver), *Methol Enzymol*, 94: 154-158, 1983.

22. Fisher GJ, Talwar HS,Tavakkol A et al.: Phosphoinositide-mediated signal transduction in normal and

psoriatic epidermis, *J Invest Dermatol*, **95**: 15S-17S, 1990.

23. Goldstein IM: Agents that interfere with arachidonic acid metabolism. In: *Inflammation: Basic Principles and Clinical Correlates* (Gallin JI, Goldstein IM, Snijderman R, ed.) New York: Raven Press: 935-945, 1988.

24. Crabtree GR: Contingent genetic regulatory events in T lymphocyte activation, *Science*, **243**: 355-361, 1989.

25. Wertz PW, Downing DT: Free sphingosine in human epidermis, J Invest Dermatol, **94**: 159-161, 1990.

Chapter IV

INHIBITION OF EPIDERMAL ODC INDUCTION BY LIGANDS OF THE STEROID HORMONE RECEPTOR SUPERFAMILY

4.1 THE INDUCTION OF EPIDERMAL ODC FOLLOWING TAPE STRIPPING IS INHIBITED BY A TOPICAL VITAMIN D3 ANALOGUE (MC903)

W.P. Arnold, P.C.M. van de Kerkhof

This work was presented at the 18th World Congress of Dermatology, New York, June 12th - 18th, 1992

Topical application of vitamin D3 derivatives has been shown to be effective in the treatment of psoriasis. Interference with the state of epidermal hyperproliferation has been suggested to be a major target for the action of vitamin D3 analogues. In this respect ornithine decarboxylase (ODC) is an enzyme with a key role in epidermal homeostasis.

The aim of the present study was to investigate the effect of MC903, a vitamin D3 analogue, on ODC levels in the psoriatic lesion as a state of hyperproliferation and to examine its influence on the induction of hyperproliferation by tape stripping.

In total 15 patients with chronic plaque psoriasis were treated in a double blind approach with MC903 in two different cream bases or a placebo cream. In addition to lesional skin a test area of uninvolved skin was treated with the cream twice daily. Keratotome biopsies were taken from the lesions before and after 8

weeks treatment and from the stripped areas, pretreated and untreated symptomless skin.

Although ODC activity within the MC903 treated lesions remained unaffected, the trauma induced induction of ODC activity proved to be profoundly inhibited by MC903. The observation that this vitamin D3 analogue inhibits the induction of ODC in vivo lends further support to the hypothesis that the steroid receptor superfamily is of general relevance for epidermal proliferation control in humans.

INTRODUCTION

The skin is the site for the synthesis of vitamin D and moreover a target tissue for its active metabolite, 1,25-(OH)2 D3. This hormone inhibits proliferation and induces terminal differentiation of the epidermis in vitro and in vivo. (reviewed by Holick)[1] Therefore topical and oral administration of 1,25-(OH)2 D3 have been used successfully for the treatment of psoriasis, a hyperproliferative skin disorder with incomplete differentiation.[1]

1,25-(OH)2 D3 is also a calcium regulating hormone, so its administration may result in development of hypercalcaemia and hypercalciuria. MC903 is a novel 1,25-(OH)2 D3 analogue which is as potent as 1,25-(OH)2 D3 in the epidermis, but is at least 100 times less active in its effects on calcium metabolism.[2] MC903 in an ointment base has proven to be an effective and safe

treatment for psoriasis in several double-blind studies.[3,4,5]

Ornithine decarboxylase (ODC) is a rate-limiting enzyme for polyamine biosynthesis. Polyamines are required before DNA synthesis can begin, thus ODC is an appropriate "marker enzyme" for cellular proliferation.[6] In normal epidermis ODC levels are undetectable, but ODC induction has been demonstrated following injury (tape stripping[7], UV-radiation[8]) and application of tumor promoting agents.[9] Stripping of human epidermis in vivo causes an ODC peak at 8 h.[10] Several groups have reported increased ODC levels in the lesions of psoriasis, and have shown that these normalize following therapy (reviewed by Lowe).[11]

1,25-$(OH)_2$ D_3 as well has proven to inhibit the ODC induction in the epidermis of rodents,[12,13] but to the best of our knowledge not yet in human epidermis. Therefore the aim of the present study was to investigate the effect of MC903 on ODC levels in the psoriatic lesion as a state of chronic hyperproliferation, and to examine its influence on the induction of hyperproliferation by tape stripping.

Subjects and treatment protocol

Fifteen patients with chronic plaque psoriasis (8 M, 7 F; age range 18-75, mean 39 year) collaborated for 10 weeks in a double blind study to evaluate the efficacy of two different MC903 cream formulations, as part of a large European study undertaken by Leo Pharmaceutical Products, Denmark. In cream "A" MC903 was dissolved with an emulgator and in cream "B" MC903 was suspended as fine particles, both at 50 µg/g.

All patients underwent a preliminary "wash out" period of 2 weeks (treatment of the lesions with an indifferent cream). Six patients were randomly allocated a supply of cream A, 6 of cream B and three the placebo. The cream was applied to all lesions (other than those on the scalp, face or anogenital region) twice daily for a period of 8 weeks. No other antipsoriatic therapy was permitted, except for the localizations mentioned above.

Blood samples for analysis of Hb, RBC, WBC, platelets, bilirubin (total), alkaline phosphatase, alanine aminotransferase, creatinine, calcium (total), albumin and phosphate were taken after 0, 2 and 8 weeks treatment.

Skin sampling and Sellotape stripping

Epidermal slices of approximately 1 cm^2 were taken from the centre of a lesion on the right elbow on the first and last day of the MC903 treatment. Biopsies were

cut from sites still showing some psoriatic activity. For this we used a Castroviejo keratotome (Storz Instr. Co.) set for a depth of 0.3 mm.

In addition, the patients were asked to apply the cream on an uninvolved area of 5 cm^2 of their left upper arm in the last 3 weeks. On the last day this treated uninvolved area and the opposite untreated area on the right arm were stripped with Sellotape^R as described previously.[14] After 8 h a keratotome biopsy was taken from both sites. All biopsies were snapfrozen in liquid nitrogen and stored at -80°C prior to analysis.

ODC and protein measurement

Measurement of ornithine decarboxylase and soluble protein was done as described previously.[10] In brief, shave biopsies were homogenised in buffer and aliquots were incubated at 37°C after adding L-[1-^{14}C]-ornithine. Each tube was fitted with a plastic cap carrying a filter paper moistened with KOH. The reaction was stopped with Hcl, again incubated and $^{14}CO_2$ in the paper was measured. Soluble protein was determined in the clear supernatant by direct fluorescence.

Statistical analysis

Analysis of variance was carried out for treated and untreated skin using the SAS package (t-test for paired data) and a VAX computer system.

Treatment with MC903 cream for 8 weeks caused virtual clearing in three patients, five patients showed a moderate reduction of their lesions and in three patients the lesions remained in a steady state. One patient dropped out due to the use of a solarium in the trial period. In the placebo group (n=3) all psoriatic lesions extended. No pathological changes with respect to haematology and blood chemistry were seen during the trial period. These data will be included in the joint European Study to be reported in full by Leo Pharmaceutical Products in due course.

In the ODC assay the reagent blank (including instrument background) averaged about 22 cpm; total reaction samples were between 60 and 950 cpm. The assay was linear with respect to time for about 45 min, so an incubation period of 45 min was used for routine measurements. The reproducibility was good, duplicate assays averaging within ± 4.1% of the mean.

In the psoriatic lesion ODC levels before treatment (n=13) were 2.6 ± 0.7 pmol/min/mg protein (mean ± SEM). This is in agreement with a previous report of Russell et al.,[15] who found ODC levels of 2.7 pmol/min/mg protein. ODC levels after treatment with MC903 (n=11) in creams A and B were 3.1 ± 1.3 and 2.4 ± 0.6 pmol/min/mg protein, respectively. ODC values after placebo treatment (n=2) were 3.2 and 6.2 pmol/min/mg protein. Thus the present study did not show any downward regulation of ODC to basal (undetectable) levels.

In the uninvolved untreated skin ODC levels 8 hours after stripping were 58.6 ± 12.6 pmol/min/mg protein (n=11). These values are in line with our previous observations.[10] MC903 treatment (n=9) resulted in a substantial reduction, reaching 26.6 ± 6.8 in the cream A group (p = 0.06) and 17.3 ± 3.4 pmol/min/mg protein in the cream B group (p = 0.07). Pooling of all treated samples yields a value of 22.5 ± 4.2 pmol/min/mg protein, showing a highly significant difference from the controls (p=0.004). ODC values after placebo treatment (n=2) were 137.2 and 80.4 pmol/min/mg protein, excluding any base influence.

DISCUSSION

Although MC903 treatment resulted in a clinical improvement of 8 out of 11 patients, pretreatment and posttreatment ODC levels were comparable. In contrast, MC903 treatment of uninvolved skin resulted in a pronounced inhibition of the induction of ODC following standardized injury.

MC903 has proven to inhibit DNA synthesis,[2,16] to reduce the amount of keratin 16, a marker for hyperproliferation,[16,17] and to reduce the number of cycling cells indicated by nuclear staining of Ki67 (antigen expressed only in dividing cells).[18] Therefore the dissociation between clinical resolution and ODC levels in the psoriatic lesion during MC903 treatment is intriguing. It is feasible that other mechanisms are involved in the resolution of established

hyperproliferation in the chronic plaque. In this respect it is of interest that interference with inflammation control i.e. disappearance of polymorphonuclear leucocytes has been observed after 1 week's treatment with MC903.[18] On the other hand ODC might be an indicator for "complete" healing.

Trauma-induced hyperproliferation is an acute event; 8 h after trauma there is a maximal ODC induction[10] and 48 h later the percentage cells in the SG2M-phase reaches maximum levels.[14] As such this model is appropriate to study the induction of hyperproliferation in the epidermis. Remarkably this induction is blocked profoundly by MC903, which supports the hypothesis that the steroid receptor superfamily is of relevance for the control of epidermal homeostasis. Members of this family are for example the glucocorticoid-, thyroid hormone-, retinoic acid- and vitamin D- receptors. In their inactive state they are thought to be bound to an inhibitor protein that blocks the DNA-binding domain of the receptor. The binding of hormone to the receptor causes the inhibitor protein to dissociate, thereby activating the receptor. The receptor in turn is then capable of blocking or stimulating an enhancer/promoter region before or after a coding DNA-sequence, for example for ODC-mRNA.[19] However, evidence has very recently been presented for an alternative concept.[20] The phosphorylated c-jun/c-fos product (AP-1), which is responsible for activation of the ODC-promoter, complexes with and is blocked by the activated corticosteroid receptor.
In this way glucocorticoids block the ODC induction in the epidermis,[21] thyroid hormone stimulates the proliferation of epidermal cells,[22] probably via ODC

induction,[23] retinoic acid inhibits the ODC induction in the skin[7] and vitamin D3 blocks the epidermal ODC induction as well.[12,13]

The present study demonstrates the in vivo relevance of a vitamin D3 analogue on epidermal growth control of human skin. As 1,25-(OH)2 D3 has been shown to inhibit ODC induction at the gene transcript level,[24] it is worthwhile to further elucidate this mechanism in genetic terms.

REFERENCES

1. Holick MF. Skin as the site of vitamin D synthesis and target tissue for 1,25-dihydroxy vitamin D3. *Arch Dermatol* 1987; **123**: 1677-83.
2. Binderup L, Bramm E. Effects of a novel vitamin D analogue MC903 on cell proliferation and differentiation in vitro and on calcium metabolism in vivo. *Biochem Pharmacol* 1988; **37**: 889-95.
3. Kragballe K, Beck HI, Sϕgaard H. Improvement of psoriasis by a topical vitamin D3 analogue (MC903) in a double blind study. *Br J Dermatol* 1988; **119**: 223-30.
4. Staberg B, Roed-Petersen J, Menné T. Efficacy of topical treatment in psoriasis with MC903, a new vitamin D analogue. *Acta Derm Venerol* 1989; **69**: 147-50.
5. Kragballe K. Treatment of psoriasis by the topical application of the novel cholecalciferol analogue calcipotriol (MC903). *Arch Dermatol* 1989; **125**: 1647-52.
6. Lowe NJ. Epidermal ornithine decarboxylase, polyamines, cell proliferation and tumor promotion. *Arch Dermatol* 1980; **116**: 822-25.
7. Bouclier M, Shroot B, Dionisius V, Isnard JL, Hensby CN. A rapid and simple test system for the evaluation of the inhibitory activity of topical retinoids on sellotape stripping induced ODC activity. *Dermatologica* 1984; **169**: 242.

8. Lowe NJ, Verma AK, Boutwell RK. Ultraviolet light induces epidermal ornithine decarboxylase activity. *J Invest Dermatol* 1978; **71**: 417-18.

9. Lichti U, Patterson E, Yuspa SM. Retinoic acid differentially inhibits the induction of ODC by TPA and by germicidal ultraviolet light. *Proc Am Assoc Cancer Res* 1979; **20**: 105.

10. Arnold WP, van Hooijdonk CAEM, Mier PD, van de Kerkhof PCM. Induction of ornithine decarboxylase following sellotape stripping in normal and psoriatic skin. *Br J Dermatol* 1990;**123**:581-85.

11. Lowe NJ. Cutaneous polyamines and their biosynthetic enzymes. In: *Psoriasis* (Roenigk HH, Maibach HI, eds), New York: Dekker, 1985; 249-54.

12. Chida K., Hashiba H, Suda T, Kuroki T. Inhibition by 1α, 25- dihydroxy vitamin D3 of induction of epidermal ornithine decarboxylase caused by TPA and teleocidin B. *Cancer Res* 1984; **44**: 1387-91.

13. Chida K, Hashiba H, Fuhushima M, Suda T, Kuroki T. Inhibition of tumor promotion in mouse skin by 1,25-dihydroxy vitamin D3. *Cancer Res* 1985; **45** 5426-30.

14. Van de Kerkhof PCM, van Rennes H, de Grood RM, de Jongh GJ, Bauer FW, Mier PD. Response of the clinically uninvolved skin of psoriatic patients to standardized injury. *Br J Dermatol* 1983; **109**: 287.

15. Russell DH, Combert WL, Duell EA, Stawiski MA, Anderson TF, Voorhees JJ. Glucocorticoid inhibits elevated polyamine biosynthesis in psoriasis. *J Invest Dermatol* 1978; **71**: 177-81.

16. De Mare S, de Jong EGJM, van de Kerkhof PCM. DNA content and Ks 8.12 binding of the psoriatic lesion during treatment with the vitamin D3 analogue MC903 and betamethasone. *Br J Dermatol* 1990; 123: 291-95.

17. Holland DB, Roberts SG, Russell A, Wood EJ, Cunliffe WJ. Changes in epidermal keratin levels during treatment of psoriasis with the topical vitamin D3 analogue MC903. *Br J Dermatol* 1990; **122**: 284.

18. De Jong EMGJ, van de Kerkhof PCM. Simultaneous assesment of inflammation and epidermal proliferation in psoriatic plaques during longterm treatment with the vitamin D3 analogue MC903: modulations and interrelations. *Br J Dermatol* 1990; in press.

19. Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. Signaling mediated by intracellular receptors: mechanism of steroid hormone action. In: *Molecular biology of the cell*, New York & London: Garland Publ., 1989; 690-93.

20. Schüle R, Rangarajan P, Kliewer S et al. Functional antagonism between oncoprotein c-jun and the glucocorticoid receptor. Cell 1990; **62**: 1217-26.

21. Lowe NJ, Breedings J. Anti-inflammatory drug effects on ultraviolet light induced epidermal ornithine decarboxylase and DNA synthesis. *J Invest Dermatol* 1980; **74**: 418-20.

22. Holt PJA. In vitro responses of the epidermis to triiodothyronine. *J Invest Dermatol* 1978; **71**: 202-04.

23. Ruel J, Chenard C, Coulombe P, Dussault JH. Thyroid hormones modulate ornithine decarboxylase in the immature rat cerebellum. *Can J Physiol Pharmacol* 1984; **62** 1279-83.

24. Rius C, Aller P. Modulation of ornithine decarboxylase gene transcript levels by differentiation inducers in human promyelocytic leukemia HL 60 cells. *Cell Diff Develop* 1989; **28**: 39-46.

4.2 THE INDUCTION OF EPIDERMAL ODC FOLLOWING UV-B IRRADIATION IS INHIBITED BY ESTRIOL

W.P. Arnold, B.J.H. Pennings, P.C.M. van de Kerkhof

This work was presented at the 23rd annual meeting of the E.S.D.R., Amsterdam, April 3rd - 6th, 1993

The induction of epidermal ornithine decarboxylase (ODC) can be partially blocked by corticosteroids, retinoic acid or active vitamin D_3. The influence of the other members of this so-called "steroid hormone receptor superfamily" (SHRS), namely the sex-steroids and thyroid hormone, is unknown in epidermis, but they enhance ODC induction in certain other tissues.

Here we investigated whether topical estriol leads to a spontaneous and/or enhanced epidermal ODC induction 8 h after UV-B irradiation of 6 postmenopausal women. Contrary to expectation, estriol did not stimulate induction, but reduced induction by 44%. This observation raises the possibility that all members of the SHRS may share a common AP-1 binding site.

INTRODUCTION

Ornithine decarboxylase (ODC) is the rate-limiting enzyme in the production of

polyamines, which are essential for DNA duplication. Hence, ODC is used as "marker-enzyme" for proliferation. Under physiological circumstances epidermal ODC is biochemically not detectable, but can be induced following standardized injuries such as tape stripping of the stratum corneum (activity peak after 8 h)[1] or irradiation with UV-B (peak after 24 h).[2] This induction can be partially blocked by certain members of the so-called "steroid hormone receptor superfamily", namely corticosteroids,[1] retinoic acid[3] and active vitamin D_3.[4] The influence of other members, the sex-steroids (progesterone, estrogens, testosterone) and thyroid hormone has not yet been investigated in the epidermis, although they enhance ODC induction is other tissues.[5,6,7] Since corticosteroids, retinoic acid and calcipotriol are potent antipsoriatic drugs, it seems worthwhile to study the effects of the other members of this superfamily on epidermal ODC induction.

Human keratinocytes contain estrogen receptors in varying concentrations, apparently depending on location and age.[8,9,10] However, exact information is not available due to limitations in detection techniques. In the epidermis estrogens have once been reported to increase the mitotic rate,[11] but this single early report has remained unconfirmed. They have negative effects on epidermal adnexal structures, i.e. by reducing the size and activity of sebaceous glands[12] and the rate of hair growth.[13] In the dermis estrogens stimulate the synthesis, maturation and turn over of collagen,[14] increase the synthesis of hyaluronic acid[15] and increase the vascularization.[16]

Here we have addressed the following question: Does topical application of estriol lead to a spontaneous and/or enhanced induction of ODC in human epidermis *in vivo*? ODC measurements were carried out in vehicle- and estriol-treated epidermis 8 h after

irradiation with UV-B, when ODC activity is still suboptimal, and in estriol treated epidermis only.

METHODS

Subjects Six postmenopausal women (age range 57-69 years) without estrogen substitution therapy (tablets, plasters or vaginal creams) and without a previous history or signs of uterine-/breastcancer, liver pathology or thrombo-embolic processes participated in this project. Every woman had skin type II or III. Informed consent was obtained from all the subjects and the study had the approval of the Medical Ethics Committee.

The minimal erythema dose (MED) for each individual was determined and two sites on their backs (3 cm^2) were each irradiated with 3 MEDs of UV-B according to MacKenzie. Immediately after irradiation the left site was treated with 200 mg cremor lanette (per g: lanette wax cream 150 mg, cetiol 200 mg, sorbitol in water), the right side with the same amount of estriol (Sigma) in cremor lanette (1 mg/g) and this estriol cream was also applied on an unirradiated site on the right side. All three areas were occluded with a non-toxic plastic foil for 8 h. After this period the skin was cleaned with 70% ethanol and biopsies (approximately 1 cm^2) were taken from the centre of each site using a Castroviejo keratotome (Storz) under local anaesthesia produced by cooling the skin with ethyl chloride. Biopsies were washed briefly in cold phosphate buffered saline and snap frozen in liquid nitrogen prior to analysis.

ODC and protein measurements These have been described previously;[1] the ODC assay

was modified by reducing the amount of "cold" ornithine in the reaction mixture from 40 nmol to 4 nmol to improve the sensitivity.

Statistics The Wilcoxon signed-ranks test for matched pairs (two-tailed) was used for statistical analysis.

RESULTS

Although all women received the standardized dose of 3 MED of UV-B, the range of ODC induction varied from 8 to 65 pmol/min/mg protein, as shown in Table I. These findings were in accordance with previous observations that an increase in age is accompanied with an increased spread in individual values (unpublished data). Therefore we normalized all ODC activities to 100% (see Table I, col. 6).

At the time of biopsy no differences in erythema were visible in the vehicle- and estriol-treated irradiated areas. Nevertheless, as seen in Table I, ODC activities in the estriol-treated irradiated sites of all six women were lower than the vehicle-treated irradiated areas, averaging 56% $\pm$ 10% (mean $\pm$ SEM; p=0.03) of this control, almost in accordance with the effects of a potent corticosteroid[1]. ODC activities in the estriol-treated sites without irradiation were not detectable in any of the 6 samples.

LEGEND TO TABLE I

ODC levels following UVB only, UVB plus estriol and estriol only. The figures in col. 6

are calculated as (col. 4/col. 3) x100%.

Subject No	Age	ODC (pmol/min/mg protein)			Effect of estriol
		UV-B only	UV-B + estriol	estriol only	
1	69	08	03	< 1	38%
2	63	17	05	< 1	29%
3	62	49	34	< 1	69%
4	57	38	31	< 1	82%
5	60	65	55	< 1	85%
6	62	29	10	< 1	34%
Mean	62	34	23	< 1	56%
SEM	1.6	8.5	8.4	-	10%

DISCUSSION

It is clear from our results that topical application of estriol does not lead to a spontaneous or enhanced induction of ODC in human epidermis *in vivo*. On the contrary, this member of the steroid hormone receptor superfamily seems to share the capacity to antagonize ODC transcription with its corticosteroid-, retinoic acid- and vitamin D_3-relatives.

Activated corticosteroid receptors reduce ODC induction by formation of a

heterodimer with the ODC-transcription factor AP-1,[17] probably subsequent to binding onto their specific response elements immediately upstream of the coding region of the DNA. Similar mechanisms are thought to be responsible for ODC inhibition by retinoic acid and vitamin D_3.[18] Could it be that this AP-1 binding site already existed in the phylogenetically oldest steroid receptor molecule and has remained constant during evolution, adding new hormone- and DNA-binding regions at different sites?[19] But how could the opposite effects of estrogen on ODC induction in epidermis as compared to other tissues be explained? An explanation may be that in these latter tissues ODC transcription is directly regulated by the activated estrogen-receptor, without participation of the c-jun/c-fos complex.

Much research still has to be done. For example, it would obviously be of value to quantify and characterize estrogen binding sites; unfortunately using current techniques this requires more material than can conveniently be obtained from biopsies. Secondly, modern flowcytometric methodology offers the possibility to re-investigate the early claim regarding the influence of estrogens on epidermal cell cycle kinetics. Finally, localization, isolation and synthesis of this common AP-1 binding site, may eventually lead to a strongly antiproliferative drug without hormonal side-effects.

ACKNOWLEDGEMENTS

We gratefully acknowledge Prof.Dr. A. Smals, Drs. P.G. Koenders and D. van Tienoven (Dept. of Endocrinology, University Hospital Nijmegen) for support of this study, Drs. Ch.

Kremer (Nourypharma BV, Oss, The Netherlands) for preparing the creams, Prof.Dr. P.D. Mier (Dept. of Dermatology, University Hospital Nijmegen) for critical reading of the manuscript and Miss Z. Arts for typing this manuscript.

REFERENCES

1. Arnold WP, van Hooijdonk CAEM, Mier PD, van de Kerkhof PCM: Induction of ornithine decarboxylase following sellotape stripping in normal and psoriatic skin. Br J Dermatol 123: 581-585, 1990.

2. Lowe NJ, Verma AK, Boutwell RK: Ultraviolet light induces epidermal ornithine decarboxylase activity. J Invest Dermatol 71: 417-418, 1978.

3. Lichti U, Patterson E, Yuspa SM: Retinoic acid differentially inhibits the induction of ODC by TPA and by germicidal ultraviolet light. Proc Am Assoc Cancer Res 20: 105, 1979.

4. Arnold WP, van de Kerkhof PCM: The induction of epidermal ornithine decarboxylase following tape stripping is inhibited by a topical vitamin D3 analogue (MC 903). Br. J Dermatol 125: 6-8, 1991.

5. Lavia LA, Stohs SJ, Lemon HM: Polyamine biosynthetic decarboxylase activities following estradiol-17 beta or estriol stimulation of the immature rat uterus. Steroids 42: 609-618, 1983.

6. Janne O: Androgen receptor-dependent regulation of ornithine decarboxylase gene expression in mouse kidney. *In: Steroid Hormone Receptors (Erikson, Gustafson, eds)* 461-490, 1983.

7. Ruel J, Chenard C, Coulombe P, Dussault JH: Thyroid hormones modulate ornithine decarboxylase in the immature rat cerebellum. Can J Physiol Pharmacol 62: 1279-1283, 1984.

8. Hasselquist MB, Goldberg N, Schroeter A, Spelsberg TC: Isolation and characterization of estrogen receptor in human skin. J Clin Endocrin Metab 50: 76-82, 1979.

9. Punnonen R, Lövgren T, Kouvonen I: Demonstration of estrogen receptors in the skin. J Endocrinol

Invest 3: 217-221, 1980.

10. Schmidt JB, Lindmaier A, Spona J: Hormone receptors in pubic skin of premenopausal and postmenopausal females. Gynecol Obstet Invest 30: 97-100, 1990.

11. Bullough WS: Hormones and mitotic activity. Vitam Horm 13: 261-290, 1955.

12. Ebling FJ: The effects of cyproterone acetate and oestradiol upon testosterone stimulated sebaceous activity in the rat. Acta Endocrinol (Kbh) 72: 361, 1973.

13. Davis BK: Quantitative morphological studies upon the influence of the endocrine system on the growth of hair by white mice. Acta Endocrinol (Kbh) 44: 9, 1963.

14. Hennman DH: Effect of estrogen on *in vivo* and *in vitro* collagen biosynthesis and maturation in old and young female guinea pigs. Endocrinology 83: 678, 1968.

15. Sobel H, Lee KD, Hewlett MJ: Effect of estrogen on acid glycosaminoglycans in skin of mice. Biochem Biophys Acta 101: 225, 1965.

16. Reynolds SRM, Foster FI: Peripheral vascular action of estrogen observed in the ear of the rabbit. J Pharmacol Exp Ther 68: 173, 1940.

17. Schüle R, Rangarajan P, Kliewer S et al.: Functional antagonism between oncoprotein c-jun and the glucocorticoid receptor. Cell 62: 1217-1226, 1990.

18. Schüle, Umesono K, Mangelsdorf DJ et al.: Jun-fos and receptors for vitamins A and D recognize a common response element in the human osteocalcin gene. Cell 61: 497-504, 1990.

19. Lenard J: Mammalian hormones in microbial cells. TIBS 17: 147-150, 1992.

Chapter V

ODC AS MARKER ENZYME TO TEST (NEW) ANTI-PSORIATIC THERAPIES

5.1 EFFECTS OF SPHINGOSINE, ISOQUINOLINE AND TANNIC ACID ON THE HUMAN TAPE-STRIPPING MODEL AND THE PSORIATIC LESION

W.P. Arnold, C.P. Glade, P.D. Mier, P.C.M. van de Kerkhof

Parts of this work were presented at the 22nd annual meeting of the E.S.D.R., London, April 4th - 7th, 1992

Published data (mainly from rodent skin) suggest a correlation between compounds which inhibit protein kinase C (PKC), have anti-inflammatory or anti-tumour promoting characteristics and possess antipsoriatic potential. Here we have investigated the effects of topical application of sphingosine (Sph; a naturally occurring PKC-inhibitor), isoquinoline (IQ; a component of coal tar which showed antipsoriatic capacities in the mouse tail model) and tannic acid (TA; a plant phenol with anti-tumour promoting activity) to human skin. In each case we have assessed a) the level of induction of ornithine decarboxylase (ODC) following Sellotape^{R}-stripping as an indicator for potential PKC inhibition *in vivo*, and b) its effects on the lesions of chronic plaque psoriasis.

The control group consisted of 18 healthy volunteers, used for the ODC induction experiments (0.0/0.1/0.2M Sph in ethanol, 100% coal tar and 0/50mM TA in acetone) and 17 psoriatic patients used for double-blind scoring of two randomly selected lesions (0.0/0.1M Sph in ethanol, 0.0/0.2% IQ in white vaseline / lanette wax cream 50%/50%

and 0/10% TA in lanette wax cream) and also for certain of the ODC induction experiments (0.0/0.2% IQ and 0/10% TA). Biopsies were taken 8 h after stripping and ODC activity was assessed by measurement of $^{14}CO_2$ release. Lesions were scored with a modified Psoriasis Area and Severity Index on days 0, 7 (IQ and TA), 13 (Sph) and 21 (IQ and TA).

Application of 0.1M or 0.2M Sph resulted in a decrease of ODC activity of 52 % and 66 % respectively ($p < 0.01$), but histologic sections showed intra-epidermal necrosis. IQ, coal tar and TA did not have any significant influence on ODC induction ($p > 0.05$). All compounds failed to show significant improvement of the psoriatic lesions. Therefore we may conclude that 'theoretical' anti-psoriatic agents may be limited in practice by cytotoxicity and hence a narrow therapeutic index, poor penetration and lack of specificity. Further, a marked difference between the effects of these compounds on the skin of different species increases the difficulty of predicting antipsoriatic activity.

INTRODUCTION

Protein kinase C (PKC) is a calcium- and phospholipid-dependent serine/threonine kinase of central importance in the signal transduction pathways which mediate cellular responses to inflammatory stimuli.[1] It is immediately downstream to the phosphotidylinositol cycle, being activated by diacylglycerol and Ca^{2+}. PKC in turn mediates a number of subsequent events, including initiation of the arachidonic acid cascade, phosphorylation of the Na^+/H^+ pump and the induction of many gene products (such as the enzyme ornithine

decarboxylase, ODC[2]) via activation of the transcription factor AP-1.[3] It has become clear that activation of PKC is necessary for the recruitment of quiescent keratinocytes into the cell cycle,[4] and there is strong evidence that it is chronically over-stimulated in the lesions of psoriasis.[5] Experimentally, PKC is activated by phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA); topical application of these compounds causes an acute inflammatory response and, following repeated application, results in tumour promotion.[1,2]

The foregoing data would lead us to expect some correlation between the ability of a compound to inhibit PKC, anti-inflammatory or cytostatic working, anti-tumour promotion characteristics and antipsoriatic potential. In fact, a number of compounds have been reported in which at least two of these effects have been described; for example the well-known antipsoriatic agent dithranol has recently been reported to be a potent PKC inhibitor.[6] However, little systematic work has been carried out along these lines. Further, the available data are obscured by uncertainties regarding species differences; this problem is exacerbated by the fact that no reliable animal model is available for psoriasis, and the experimental application of many chemical substances (especially TPA) to human skin is ethically unwarranted.

In this report we have investigated the effect of topical application of three compounds to human skin, which are either established PKC inhibitors or known anti-tumour promoters. These substances were sphingosine[7] (a naturally occurring lipid-metabolite[8],which direct absorption has been demonstrated in intact mouse-skin), isoquinoline[9,10] (0.2% present in the therapeutically accepted agent coal tar[11]) and tannic

acid[12] (earlier used, for example, following burns[13]). In each case we have assessed a) the level of ODC induction following Sellotape^{R} stripping[14] as an indicator for potential PKC inhibition *in vivo*, and b) its effect on the lesions of chronic plaque psoriasis.

METHODS

Subjects

The control group consisted of 18 paid volunteers (6 male, 12 female; age range 18-57 years, mean 28 years) without signs or history of skin diseases. Areas on their back were treated as described in the treatment protocols; sub-group numbers are given in table I.

The psoriatic group comprised 17 paid patients (11 male, 6 female; age range 29-64 years, mean 44 years) with chronic plaque psoriasis, who had received no oral/topical therapy for at least 4/2 weeks prior to investigation respectively. In each patient two symmetrical, comparable lesions were selected at a distance of at least 5 cm from other psoriatic areas. Lesions were treated as described in the treatment-protocols and scored double-blind with a modified Psoriasis Area and Severity Index (mPASI) : $[E + I + D]_{t=x}$ x $(\emptyset_{t=x}/\emptyset_{t=0})$. E=erythema, I=infiltration, D=desquamation; severity scale 0-4, t = time-point of scoring, x = duration of treatment (days) and $\emptyset$ = biggest measurable crosssection (cm). Scoring-days and sub-group numbers are given in table II.

The use of UV-B sources or vitamin A/D tablets was specifically excluded in all subjects immediately prior to or during the experiments. Informed consent was obtained from all subjects and the study had the approval of the Medical Ethics Committee.

Sphingosine Treatment Protocol

Volunteers. Three sites of 1.75 cm^2 each were marked and 35 µl solution of 0.2 M and 0.1 M sphingosine (Sigma) in ethanol and ethanol only were applied through a plastic cylinder. The ethanol was evaporated with air under pressure. The sites were then occluded for 30 min with a non-toxic plastic foil. Tape stripping was carried out, immediately followed by a second application of 35 µl sphingosine of the same concentrations. After being covered with a gauze for 8 h, biopsies were cut from each site.

Ethanol only and 0.2 M sphingosine were applied to the backs of 2 volunteers following the same procedure as described above. Razor blade biopsies were taken 8 h after tape stripping. Paraffin tissue sections were stained with H & E.

Patients. Using a within subject left-right comparison one lesion was treated with 0.1 M sphingosine and the contralateral lesion with ethanol only. Volumes of 20µl/cm^2 were applied twice a day for 12 days and the treated areas were covered with a gauze.

Isoquinoline/Coal Tar Treatment Protocol

Volunteers. Crude coal tar (GEM Pharma) containing 0.2% isoquinoline, was applied in an area of 3 cm^2 and covered with a gauze. This area and a contralateral site were tape stripped after 16 h, immediately followed by a second application of the coal tar on this first area, which was again covered with a gauze. A biopsy was taken from both sites after 8 h. Additionally 2 volunteers underwent the same treatment protocol as for the uninvolved skin of the psoriatic patients, i.e. twice daily applications of coal tar for one week, before tape-stripping and biopsy.

Patients. On day 0 two jars with cream were supplied; one jar contained vaseline album / lanette wax cream 50% / 50% with 0.2% isoquinoline (Sigma) and the other jar the basecream only. The jars were randomly allocated to "left" or "right" and the patients were asked to use the cream according this division on their left or right lesion twice daily. In their last trial week the patients started applying the creams also twice daily on 2 uninvolved areas of ± 3 cm^2 at least 10 cm away from the treated lesion. On day 21 these areas were tape stripped, immediately followed by application of the corresponding cream, occlusion with a gauze and after 8 h a biopsy from each site was taken.

Tannic Acid Treatment Protocol

Volunteers. Thirty µl of 50 mM tannic acid (Sigma) in acetone and acetone only were applied on 2 areas of 1.75 cm^2, evaporated with air under pressure and occluded with a non-toxic plastic foil. Tape stripping of these areas was carried out after 1 h. Immediately after this procedure 30 µl of the same solution was applied to these sites, which were left under occlusion with a non-toxic foil for 8 h. Biopsies were then taken.

Patients. On day 0 two jars were supplied; one jar contained lanette wax cream with 10% tannic acid and the other jar lanette wax cream only. The jars were randomly allocated to "left" or "right", following the same treatment protocol as for isoquinoline.

Tape Stripping & Biopsies

After cleaning the skin with 70% ethanol (in the cream-experiments only), Sellotape^R stripping was done as described previously,[14] using a plastic template to restrict the

damage to the pre-treated areas only. A biopsy of approximately 1 cm^2 was cut from each site, using a Castroviejo Keratotome (Storz) set for a depth of 0.2 mm after cooling the skin with an ethyl chloride spray. Biopsies were washed briefly in ice-cold phosphate-buffered saline and snap frozen in liquid nitrogen prior to further analysis.

ODC & Protein Measurement

Measurements of ODC activity and soluble protein levels were carried out as previously described.[14] Biopsies were homogenized in buffer (0°C) and aliquots incubated at 37°C after adding L-(1-^{14}C)-ornithine (NEN). Each tube was fitted with a plastic cap carrying a filter paper moistened with potassium hydroxide; the reaction was stopped with hydrochloric acid, incubated again and $^{14}CO_2$ was measured. Soluble protein was determined in the clear supernatant by direct fluorescence (λ_{EX}=280nm, λ_{EM}=340nm).

Statistical Analysis

Analysis of variance was carried out for treated and untreated skin using the SAS package (t-test for paired data) and a VAX computer system.

RESULTS

Induction of ODC

Table I gives an overview on the effects of sphingosine, isoquinoline and tannic acid on epidermal ODC induction. Application of 0.1 M or 0.2 M sphingosine resulted in a

decrease of ODC activity of 52% and 66% respectively. This effect was significantly different compared to ethanol only ($p<0.01$); there was no significant difference between application of 0.1 or 0.2 M sphingosine (Duncan's multiple range test, $\alpha=0.05$). However, at a macroscopical level, there was both subjective (mild burning sensation) and objective (spreading erythema) evidence of a marked dose-dependent irritant effect of sphingosine. Application of 0.2% isoquinoline or even crude coal tar did not have any significant influence on ODC induction ($p>0.05$ in both groups). These coal tar results were not altered by increasing the treatment period (n=2). The activity of ODC was also neither altered significantly by 10% or 50 mM tannic acid ($p>0.05$ in both groups) compared to basecream or acetone respectively. The substantial differences of ODC values in those two groups are presumably due to the use of occlusion in the latter, since increased temperature and humidity enhance wound-healing.

Agent	[...]	n	ODC activity in treated area	ODC activity in base-treated area	p
Sphingosine	0.1M	7	30 ± 8	62 ± 6	< 0.01
	0.2M	7	21 ± 4	62 ± 6	<0.01
Isoquinoline	0.2%	7	116 ± 18	113 ± 35	>0.05
Coal tar	100%	6	91 ± 27	115 ± 20	>0.05

Tannic acid	10%	6	71 ± 17	71 ± 7	>0.05
	50mM	4	176 ± 40	131 ± 28	>0.05

Table I ODC activities, expressed as pmol/min/mg protein (mean ± SEM), 8 h after tape stripping of (pre-)treated skin.

Histology

The histologic sections from the volunteers treated with ethanol only showed minor PMN infiltration 8h after tape stripping, with all epidermal layers (excluding the str. corneum) and the underlying tissue being completely undamaged (fig.1a). In the 0.2M sphingosine treated sections there was a slightly more profuse PMN infiltration with intra-epidermal (basal & suprabasal) vesicles filled with a necrotic, amorphous material (Fig.1b).

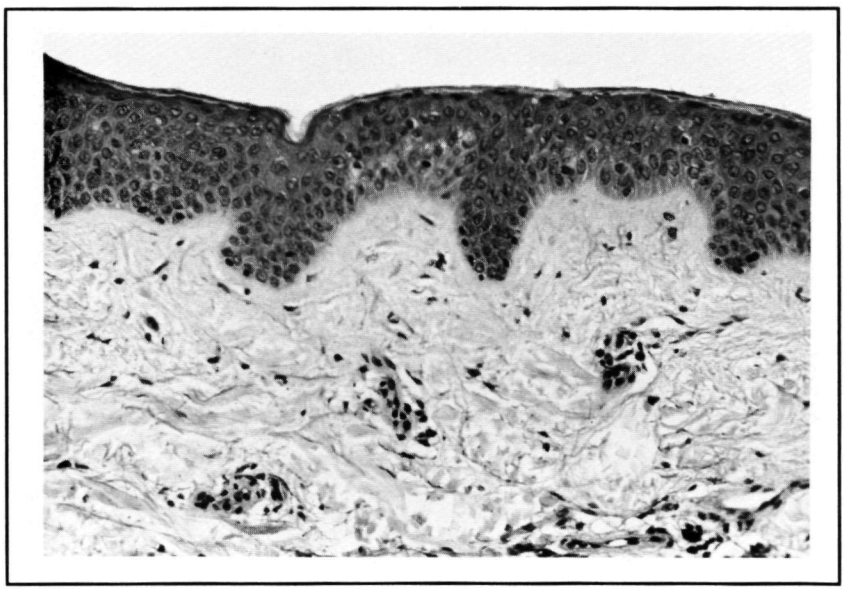

Fig. 1A

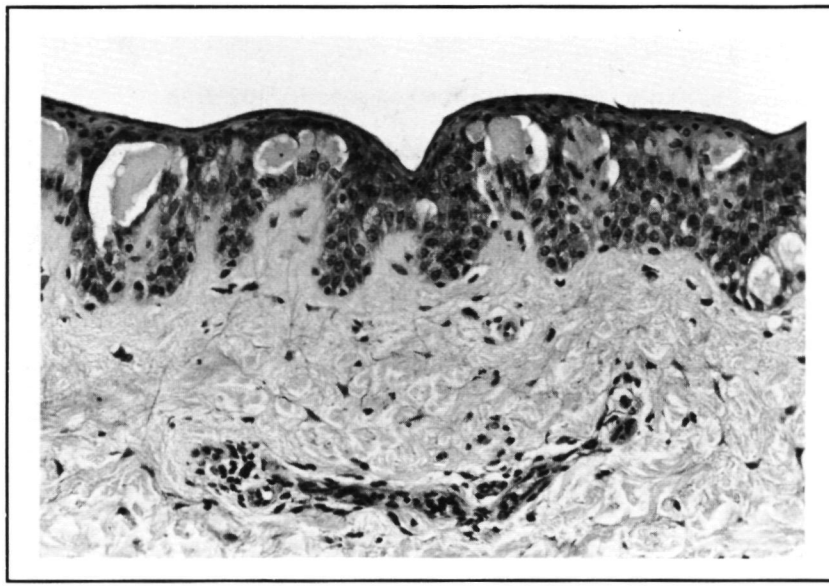

Fig. 1B

Figure 1 Histologic effects of sphingosine on tape-stripped human epidermis. (A) Ethanol only 8 h after the second application. (B) Plus 0.2 M sphingosine 8 h after the second application. Magnification 25x.

Clinical Trials

Table II gives an overview on the effects on sphingosine, isoquinoline and tannic acid on the psoriatic lesions. In these concentrations and these vehicula, all compounds failed to show significant improvement in this double-blind approach ($p>0.05$). This was in accordance with the negative subjective estimation of the patients. No side-effects of any of the drugs or basis were noticed by the physician nor mentioned by the patients.

Agent	n	day 0	day 7	day 13	day 21
Sphingosine 0.1M		7.8 ± 1.0	-	5.8 ± 1.0	-
Ethanol only	4	7.5 ± 0.9	-	6.2 ± 1.6	-
Isoquinoline 0.2%		5.6 ± 0.8	4.1 ± 0.7	-	2.1 ± 0.3
Vaseline/Lanette wax cream	7	5.4 ± 0.6	3.2 ± 0.7	-	2.6 ± 0.7
Tannic acid 10%		5.7 ± 0.7	5.2 ± 0.7	-	3.5 ± 0.6
Lanette wax cream	6	5.3 ± 0.7	4.0 ± 1.0	-	4.0 ± 0.6

Table II mPASI's (mean ± SEM) of the treated psoriatic lesions. No agent resulted in a significant difference compared to its vehiculum at any time ($p>0.05$).

DISCUSSION

Sphingosine

Three new observations are reported here. First, sphingosine at a concentration of 0.1 M or 0.2 M blocks the induction of ODC following tape stripping of human skin. Second, at this concentration there is evidence of a marked irritant effect both at the macroscopical (0.1 M and 0.2 M) and at the histological (0.2M) levels. Finally, 0.1 M sphingosine fails to show a significant effect on the psoriatic lesion.

The effects of sphingosine on ODC induction are compatible with those reported

by Gupta et al.[7] and Enkvetchakul et al.,[15] using TPA-stimulated mouse skin. Surprisingly however, neither group mentions cytotoxicity. This contrast suggests that the loss of barrier function resulting from stripping permits cytotoxic concentrations of sphingosine to reach the viable cell layers. But our preliminary experiments with 0.01 M and 0.02 M sphingosine failed to show inhibitory effects on ODC induction, so the range between PKC inhibition causing cellular effects and PKC inhibition causing cytotoxicity is extremely small. This finding is in line with data regarding various PKC inhibitors in cell culture experiments.[16] Whether this small range will diminish the therapeutic potential of PKC inhibitors in psoriasis, remains an open question for the future. Another question concerns the methodological distinguishment between inhibition of the induction or direct inhibition of ODC activity by sphingosine. However, no report of direct effect of sphingosine on enzymatic activity of ODC has ever been published.

It may be noted that sphingosine reduced ODC levels following stripping from ± 60 to ± 30 pmol/min/mg protein. Since reported ODC activity in untreated psoriatic lesions is around 3 pmol/min/mg protein[17], one may assume that sufficient activity remains to support this increased proliferation rate. One may also assume that the irritant effect of sphingosine is counteracting any therapeutic effect induced by inhibition of ODC. However, the situation seems extremely similar to that of dithranol, since in fact (a) both are irritants, (b) both inhibit PKC[6] and (c) dithranol reduces proliferation[18]. The precise relationship between these observations is still unclear, but - as we know for dithranol - does not exclude a useful antipsoriatic effect.

Isoquinoline

Although in itself not known to have inhibitory effects on PKC, derivatives of isoquinoline[9] surely do. Additionally, isoquinoline is a component of coal tar (a potent anti-psoriatic agent), that promised anti-psoriatic capacities in the mouse tail model.[10] Therefore we tested both isoquinoline and coal tar for their effects on epidermal ODC induction (for practical reasons in psoriatic uninvolved and healthy epidermis respectively, since ODC induction does not differ between psoriatics and healthy controls[14]) and isoquinoline only for anti-psoriatic potential.

Of the common negative findings, the coal tar results are especially remarkable. Almost all anti-psoriatic therapies (corticosteroids, retinoic acid, vitamin D_3) inhibit epidermal ODC induction,[17] except the UV-B-[19] and dithranol- (unpublished data) treatments, that induce ODC per se. This study shows that one of the most efficient anti-psoriatic therapies, 100% crude coal tar, neither inhibits nor induces ODC. How all these different therapeutical approaches intervening in various ways in the disturbed triangle proliferation-inflammation-differentiation finally lead to the same nett result, remains one of the most intriguing questions in psoriasis.

Tannic Acid

The high dosage of 10% tannic acid in a cream base does not have anti-psoriatic properties and does not inhibit tape stripping-induced ODC activity in human epidermis *in vivo*. Tannic acid (TA) belongs to the naturally occurring astringents, that precipitate proteins but have so little penetrability that only the surface of cells is affected.[20] So our

negative data are wholly compatible with the extremely low penetrability of the plant phenols. Therefore, the value of our experiments with tannic acid lies in the fact, that other research-workers have never mentioned the poor absorbance of tannic acid.

In the past Dutch dermatologists used TA in a cream base as UV-B protector and German dermatologists still use TA as an adjuvant in the treatment of skin burns and acute dermatitis; in the English dermatological literature TA is not mentioned. In the biochemical literature, however, TA is a well-known inhibitor of skin tumor initiation. Recently Gali et al.[12] described for the first time its anti-tumour promoting activity. Why do our results differ from those of Gali et al.? Differences in the bioavailability of TA cannot be the reason, since in the animal model the (smaller) stratum corneum remained in situ and in our model the stripping in itself afforded maximal penetration. An explanation could therefore be that TA directly reacts with and hence inactivates TPA on the skin surface; resolution of this paradox must await further research.

In summary we may conclude that the experimental demonstration of the correlations suggested in the Introduction is limited by a number of practical considerations. These include cytotoxicity (leading to a narrow therapeutic index), poor penetration of the stratum corneum and probably lack of specificity. A further problem, illustrated by our data regarding sphingosine and tannic acid, is the apparently marked difference between the effects of these compounds on the skin of different species. It is clear that a great deal of further work remains to be done, especially in view of the large number of potent and specific inhibitors of PKC, which have recently been isolated[16] and which are now

becoming commercially available.

REFERENCES

1. Nishizuka Y (1984) The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 308: 693-698
2. Verma AK, Pong RC, Erickson D (1986) Involvement of protein kinase C activation in ornithine decarboxylase gene expression in primary culture of newborn mouse epidermal cells and in skin tumour promotion by 12-O-Tetradacanoylphorbol-13-acetate. Cancer Res 46: 6149-6155
3. Karin M (1991) The AP-1 complex and its role in transcriptional control by protein kinase C. In Molecular Aspects of Cellular Regulation, Vol 6, P Cohen and G Foulkes, eds (Amsterdam: Elsevier Science Publ), pp 143-161
4. Rijzewijk JJ, Bauer FW, Boezeman JBM, Happle R, Mier PD (1988) Recruitment of quiescent (Go) cells following epidermal injury is initiated by activation of the phosphoinositol cycle. J Invest Dermatol 90: 44-47
5. Horn F, Marks F, Fisher GJ (1987) Decreased protein kinase C activity in psoriatic versus normal epidermis. J Invest Dermatol 88: 220-222
6. Hegemann L, Bonnekoh B, Fruchtman R, Müller-Peddinghaus R, van Rooijen LAA, Mahrle G (1990) Evidence that the antipsoriatic drug anthralin acts by inhibition of protein kinase C. Skin Pharmacol 3: 196
7. Gupta AK, Fisker GJ, Elder JT, Nickoloff BJ, Voorhees JJ (1988) Sphingosine inhibits phorbol ester-induced inflammation, ornithine decarboxylase activity, and activation of protein kinase C in mouse skin. J Invest Dermatol 91: 486-491
8. Gray GM, Yardley HJ (1975) Lipid compositions of cells isolated from pig, human, and rat epidermis. J Lipid Res 16: 434-440
9. Nakadate T, Yamamoto S, Aizu E, Nishikawa K, Kato R (1990) H.7, a protein kinase C inhibitor,

inhibits phorbol ester-caused ornithine decarboxylase induction but fails to inhibit phorbol ester-caused suppression of epidermal growth factor binding in primary cultured mouse epidermal cells. Mol Pharm 36: 917-924

10. Foreman MJ, Taylor M, Clark C, Devitt H, Hanlon G, Kelly I, Lukowiecki G (1985) Isoquinoline is a possible anti-psoriatic agent in coal tar. Br J Dermatol 112: 323-328

11. Collin G, Zander M (1982) Tar and pitch. In: Ullmann's encyclopedia of technical chemistry, Weinheim, Verlag Chemie 22: 411-446

12. Gali HU, Perchellet EM, Perchellet JP (1991) Inhibition of tumor promoter-induced ornithine decarboxylase activity by tannic acid and other polyphenols in mouse epidermis in vivo. Cancer Res 51: 2820-2825

13. Davidson EX (1925) Tannic acid in the treatment of burns. Surg Genecol Obstet 26: 202

14. Arnold WP, van Hooijdonk CAEM, Mier PD, van de Kerkhof PCM (1990) Induction of ornithine decarboxylase following sellotape stripping in normal and psoriatic skin. Br J Dermatol 123: 581-585

15. Enkvetchakul B, Merril AH, Jr., Birt DF (1989) Inhibition of the induction of ornithine decarboxlyase activity by 12-O-tetradecanoyl-13-acetate in mouse skin by sphingosine sulfate. Carcinogenesis 10: 379-381

16. Hunter T, Sefton BM (1991) Methods in enzymology, Vol 201-B, section II-B. Academic Press, Inc, San Diego

17. Arnold WP, van de Kerkhof PCM (1991) The induction of epidermal ornithine decarboxylase following tape stripping is inhibited by a topical vitamin D_3 analogue (MC 903). Br J Dermatol 125: 6-8

18. Willis CM, Stephens CJM, Wilkinson JD. Differential effects of structurally unrelated chemical irritants on the density of proliferating keratinocytes in 48 h patch tests reactions. J Invest Dermatol 99: 449-453, 1992

19. Lowe NJ, Verma AK, Boutwell RK (1978) Ultraviolet light induces epidermal ornithine decarboxylase activity. J Invest Dermatol 71: 417-418

20. Swinyard EA, Pathak MA (1980) Surface-acting drugs. In: A. Goodman Gilman, L.S. Goodman, and A. Gilman (ed.), The pharmacological basis of therapeutics, 6th edition Macmillan Publ. Co., pp. 951-963.

5.2 CYCLOSPORIN A DOES NOT AFFECT EPIDERMAL PROLIFERATION FOLLOWING STANDARDIZED INJURY

W.P. Arnold, M.-J.P. Gerritsen, P.C.M. van de Kerkhof

This work was presented at the 23rd annual meeting of the E.S.D.R., Amsterdam, April 3rd - 6th, 1993

Cyclosporin A (CsA) is used in the treatment of severe psoriasis, reducing both inflammation and epidermal proliferation.Its targets in the immune system are well established, but data regarding possible direct effects on keratinocytes are contradictory. In rodent skin, topical CsA blocks the induction of ornithine decarboxylase (ODC), hence reducing polyamines essential for proliferation. In psoriatic lesional skin, however, oral CsA does not influence other epidermal proliferation-associated markers. We have now assessed ODC activity following standardized injury to psoriatic uninvolved skin, combined with nuclear monoclonal binding to Ki-67, a marker for cycling cells, to ascertain the *in vivo* effect of systemic treatment with CsA on epidermal proliferation.

Ten psoriatic patients were treated with CsA for 16 weeks. Before the start and during week 16 two uninvolved areas on their back were tape-stripped in order to induce a standardized trauma. Biopsies were taken after 8 h for ODC measurements and after 48 h for Ki-67 staining. In contrast to the pronounced influence of CsA on the Psoriasis Area and Severity Index, neither parameter was altered significantly by CsA treatment.

This report excludes indirect inhibitory effects of CsA on protein kinase C (PKC), the key-enzyme in the epidermal proliferation pathway. The question why keratinocytes contain a specific receptor for CsA remains unresolved.

INTRODUCTION

Cyclosporin A (CsA) is a well known drug in the treatment of severe psoriasis, a skin disease characterized by inflammation and epidermal hyperproliferation. It is now well-established that all known effects of CsA are, in fact, mediated by the complex of this drug with its cellular binding protein, cyclophilin [1]. CsA may affect both inflammation and epidermal proliferation. In the T-cell, it has been established that CsA modulates the transcription of messenger RNA for interleukin 2 [2-4]. In addition, antigen presentation by Langerhans'cells and HLA-Dr expression by psoriatic keratinocytes is inhibited [5,6]. On the other hand it has been demonstrated that proliferation of normal human keratinocytes is inhibited in culture, although following incubation with concentrations considerably in excess of those reached in serum during treatment of psoriatic patients [7]. In particular the transition from G_0 into G_1 phase proved to be blocked [8]. In animal skin it has been shown that CsA blocked phorbol-ester- and PUVA-induced ornithine decarboxylase (ODC) activity [9,10]. ODC is the rate-limiting enzyme in polyamine biosynthesis and hence tightly involved in proliferation [11]. Regarding the situation in human skin *in vivo*, does CsA inhibit epidermal proliferation in patients with psoriasis during systemic treatment with CsA?

As a decrease of epidermal proliferation in the psoriatic plaque obviously might be the result of reduction of the inflammatory events in the psoriatic plaques, modulation of epidermal proliferation by CsA was studied in an in vivo model. For this purpose we investigated the regenerative response following standardized injury by tape stripping, in particular the recruitment of cycling cells from the resting G_0 population [12]. Ki-67 is a monoclonal antibody against a nuclear antigen present in cycling cells [13,14]. Nuclear binding with Ki-67 indicates therefore that the cell has passed the transition point from the G_0 phase into the cycling phase. It has been demonstrated that the activity of ODC is directly linked to protein kinase C (PKC) activity [15]. Stimulation and inhibition of PKC have been demonstrated to affect accordingly the degree of recruitment of cycling cells in the epidermis [16]. Therefore nuclear binding to Ki-67 and ODC activity were assessed in the present study to find out the *in vivo* effect of systemic treatment with CsA on epidermal proliferation.

METHODS

Subjects

Ten patients with severe chronic disabling psoriasis (nine male, one female) with a mean age of 45 years participated in this study after approval of the Medical Ethics Committee. Before entry, patients had not received any oral antipsoriatic therapy for at least two weeks and no specific topical treatment for at least one week. All patients started with a dose of 3 mg/kg/day of cyclosporin A and depending on the clinical results the dose was increased monthly with 1 mg/kg/day to a maximum of 5 mg/kg/day. Every month blood

samples were taken to assess plasma CsA levels and to monitor possible side-effects.

Two days before CsA therapy was started and during week 16 of the CsA treatment, Sellotape^{R} stripping was carried out at two 2 cm^2 areas of uninvolved skin on their backs. By repeated applications of Sellotape^{R}, until the surface becomes glistening, the statum corneum is removed completely in order to induce a hyperproliferative response.

After 8 h, when the induction of ODC activity is maximal [17], a biopsy (1 cm^2, 0.2 mm) out of one area was taken with a Castroviejo keratotome (Storz Instr. Co., St. Louis, MO, USA), immediately snap frozen in liquid nitrogen and stored at -80°C prior to biochemical analysis. After 48 h, when epidermal proliferation is most pronounced [18], punch biopsies (3 mm diameter) were taken from the remaining area and embedded in Tissue Tek OCT compound (Miles Scientific, Napersville, IL, USA), snap frozen in liquid nitrogen and stored at -80°C until use.

ODC and protein measurement

The measurements of ODC and soluble protein were carried out as described previously [17]. Biopsies were homogenized in a specific buffer and aliquots incubated at 37°C after adding L-(1-^{14}C)-ornithine. Each tube was fitted with a plastic cap carrying a filter paper moistened with KOH; the reaction was then stopped with HCl, incubated again and the $^{14}CO_2$ measured. The soluble protein was determined in the clear supernatant by direct fluorescence.

Ki-67 staining procedure

Sections of 6 µm were cut, air dried and fixed for 10 min in aceton/ether 60/40 vol%. As described before [18], slides were put in a phosphate-buffered stock solution and incubated for one hour with the monoclonal antibody Ki-67 (1 : 10, Dakopatts, Copenhagen, Denmark). After two bufferwashings the slides were incubated with rabbit anti-mouse immunoglobulin conjugated with peroxidase (1 : 25, RAMPO). The slides were washed twice again, pre-incubated with sodium acetate buffer (pH 4.9) and a solution of 3-amino-9-ethylcarbazole containing 0.01% H_2O_2 was added for 10 min. This was followed by another two washings in demineralized water, before the slides were counterstained with Mayers' Haematoxylin (Sigma, St. Louis, MO, USA) and mounted in glycerol gelatin. Finally, by microscopical examination, the number of Ki-67 positive nuclei per mm length of the section were counted.

Statistical analysis

The Wilcoxon signed-ranks test for two-tailed matched pairs (before and after 4 months of treatment) was used for statistical analysis.

RESULTS

Values of ODC activities and the number of Ki-67 positive nuclei before the start with cyclosporin therapy were within the range of previous findings, excluding possible errors in methodology. In contrast to the Psoriasis Area and Severity Index (PASI), neither were influenced by the use of cyclosporin; data are summarized in table I.

TABLE I (n = 10)	Before start of cyclosporin therapy	During week 16 of cyclosporin therapy	p values
PASI	20.5 ± 4.4	4.3 ± 0.6	< 0.01
ODC	106 ± 32	125 ± 35	> 0.05
Ki-67	104 ± 21	87 ± 19	> 0.05

Legend to table I

Mean PASI scores (± SEM) of involved skin compared with mean values (± SEM) of ODC activities (pmol.min^{-1}.mg protein^{-1}) and number of Ki-67 positive nuclei (per mm section length), 8 h and 48 h respectively after tape striping of uninvolved skin, before and during cyclosporin therapy.

DISCUSSION

Following systemic treatment with CsA at dosages sufficient for a pronounced clinical effect, the recruitment of cycling epidermal cells was not affected. Both nuclear binding to Ki-67 and ODC activity following tape stripping remained unchanged.

This *in vivo* investigation is at variance with the mentioned studies on keratinocytes in culture. The discrepancy might be due to a relatively insufficient bioavailability of the

drug in the epidermis of our patients. However, the dosages used in these patients were sufficient to induce a marked clinical improvement. The present observation is compatible with the study of Gottlieb et al. [19] who demonstrated that the epidermal proliferation-associated markers TGFα, IL 6 and keratin 16 were unaffected by systemic treatment with CsA.

Recently our group has confirmed that cytosolic extracts of human epidermis, and more specifically, cultured human keratinocytes contain cyclophilin [20]. Various metabolic effects of CsA on epidermis have been shown, for example inhibition of ODC activation by phorbol-esters and PUVA irradiation [9,10]. In contrast to these effects, CsA can increase the degree of phosphorylation at serine and threonine residues via inhibition of protein phosphatase 2B [21], and is thus synergic with PKC.

Systemic treatment with CsA does not inhibit epidermal proliferation *in vivo*. So far, the question why keratinocytes contain cyclophilin remains unresolved. It is remotely possible that the release of cytokines from the epidermis might be modulated by systemic CsA treatment.

REFERENCES

1. Stamnes MA, Rutherford SL, Zuker CS. Cyclophilins: a new family of proteins involved in intracellular folding. TICB 2 : 272-276, 1992
2. Shevach EM. The effects of cyclosporin A on immune system. Annu Rev Immunol 3 : 397-423, 1985
3. Crabtree GR. Contingent genetic regulatory events in T lymphocyte activation. Science 243 : 355-361, 1989

4. Defranco AL. Immunosuppressants at work. Nature 352 : 754-755, 1991

5. Teunissen MBM, de Jager MH, Kapsenberg ML, Bos JD. Inhibitory effect of cyclosporin A on antigen and alloantigen presenting capacity of human epidermal Langerhans cells. Br J Dermatol 125 : 309-316, 1991

6. Cooper KD, Baadsgard O, Ellis CN et al.. Mechanisms of cyclosporin A inhibition of antigen presenting activity in uninvolved and lesional psoriatic epidermis. J Invest Dermatol 94 : 649-656, 1990

7. Furue M, Gaspari AA, Katz SI. Cyclosporin A inhibits proliferation of normal and transformed keratinocytes. J Invest Dermatol 90 : 796-800, 1988

8. Khandke L, Krane JF, Ashinoff R et al.. Cyclosporin in psoriasis treatment. Arch Dermatol 127 : 1172-1179, 1991

9. Elder JT, Gupta AK, Fisher GJ, Voorhees JJ. Cyclosporine inhibits ODC gene expression and acute inflammation in response to phorbol ester treatment of hairless mouse skin. Transplant Proc 3 S 4 : 95-104, 1988

10. Kono T, Mizuno N, Taniguchi S et al.. Differential effects of cyclosporin A on ornithine decarboxylase activity induced by ultraviolet-B and PUVA in mouse skin. J Invest Dermatol 96 : 871-874, 1991

11. Boutwell RK, O'Brien TG, Verma AK et al.. The induction of ornithine decarboxylase activity and its control in mouse skin epidermis. Adv Enzyme Regul 17 : 89-112, 1978

12. Boezeman JBM, Bauer FW, de Grood RM. Flow cytometric analysis of the recruitment of G_0 cells in human epidermis in vivo following tape stripping. Cell Tissue Kinet 20 : 99-107, 1987

13. Gerdes J, Schwab U, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 31 : 13-20, 1983

14. Gerdes J, Lemke H, Baisch H et al.. Cell cycle analysis of a cell proliferation -associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 133 : 1710-1715, 1984

15. Verma AK, Pong R-C, Erickson D. Involvement of protein kinase C activation in ornithine

decarboxylase gen expression in primary culture of newborn mouse epidermal cells. Cancer Res 46 : 6149-6155, 1986

16. Rijzewijk JJ, Bauer FW, Boezeman JBM et al.. Recruitment of quiescent (G_o) cells following epidermal injury is initiated by activation of the phosphoinositol cycle. J Invest Dermatol 90 : 44-47, 1988

17. Arnold WP, van Hooijdonk CAEM, Mier PD, van de Kerkhof PCM. Induction of ornithine decarboxylase following sellotape stripping in normal and psoriatic skin. Br J Dermatol 123 : 581-585, 1990

18. Rijzewijk JJ, van Erp PEJ, Bauer FW. Two binding sites for Ki-67 related to quiescent and cycling cells in human epidermis. Acta Derm Venereol 69 : 512-515, 1989

19. Gottlieb AB, Grossman RM, Khandke L et al.. Studies of the effect of cyclosporine in psoriasis in vivo : combined effects on activated T lymphocytes and epidermal regenerative maturation. J Invest Dermatol 98 : 302-309, 1992

20. Mier PD, Arnold WP, van de Kerkhof PCM. Cyclosporin A and the skin. submitted (Nature)

21. Steiner JP, Dawson TM, Fotuhi M et al.. High brain densities of the immunophilin FKBP colocalized with calcineurin. Nature 358 :584-587, 1992

Chapter VI

GENERAL DISCUSSION

6.1 Major conclusions

In healthy, undamaged human epidermis, basal ODC activity is less than 1 pmol/min/mg protein. Following tape stripping, an at least 80-fold stimulation is reached at 8 h, followed by an abrupt decline to a level of about 10 pmol/min/mg protein, which remains constant for 36 h following stripping. This response does not differ between controls and psoriatic patients, which makes an etiological role of ODC in the pathogenesis of psoriasis unlikely.

In the psoriatic lesion ODC shows an activity of about 5 pmol/min/mg protein and in this pathologic situation all basal cells are cycling.[1] So this ODC level has to be sufficient to provide polyamines for maximal DNA synthesis. Ki-67 (a monoclonal antibody that binds to a nuclear antigen expressed in cycling cells) starts to increase 32 h after stripping and reaches a peak at 40 h,[2] in parallel with the curve of the percentage cells in SG_2M-phase.[3] The first wave of DNA synthesis begins around 32 h after stripping: according to our induction curve a time point with almost twice as much ODC activity than is needed for maximal DNA synthesis. Therefore it is attractive to speculate that the ODC peak 8 h after stripping is not directly associated with DNA synthesis, but may have a role in the differentiation program.

As we have seen in Chapter I, proliferation and differentiation are closely linked in epidermis and this is especially true for the synthesis of keratins, transglutaminase and involucrin. Following tape stripping, keratinocytes show changes in the pattern of keratin synthesis within 8.5 h, i.e. the appearance of keratin 6 and 16 and a reduction of

synthesis of keratin 1.[3,4] Keratin 10 starts to decrease after 16 h.[3] Alterations in the patterns of staining with anti-involucrin and antitransglutaminase are visible within 15 h.[4] Therefore it seems logical to search for an essential function of the abundance in polyamines at 8 h in these processes, an underestimated thought in the literature.[5,6,7] Providing an alternative substrate for transglutaminase[8,9] may be an explanation, since this will result in a quick "first-aid" covering of the still viable, but unprotected epidermal cells...

Proliferation is restricted to the stratum basale only and the events in the differentiation process mentioned above take place in the suprabasal layers. ODC is thought to be expressed in the stratum basale only (in normal epidermis and following injury, although even in situ hybridization techniques have not proven this with certainty.[10,11,12] Do polyamines diffuse from the stratum basale into the suprabasal layers or are they also produced by the stratum spinosum and granulosum themselves? Hence many questions still have to be answered before the role of ODC in keratinocyte maturation will become completely clear.

Measurement of the ODC peak 8 h after stripping still represents a practical approach to study epidermal proliferation, since the peak (i) indicates an exit from G_o-phase in at least cultured 3T3-cells[13] and other tissues in vivo,[14,15] (ii) parallels the dynamics of activation of PKC by TPA[16] and (iii) permits comparison of possible inhibitory effects of drugs.

Our conclusions that neither prostaglandins nor leukotrienes are essential for epidermal ODC induction in humans *in vivo*, imply that observations in humans are

difficult to extrapolate from rodent data, and other mechanisms have to play a role in the inhibition of ODC induction by corticosteroids. Further, epidermal ODC induction is not altered in patients receiving Non-Steroidal Anti-Inflammatory Drugs.

In chapter IV it has become clear that the known ligands of the steroid hormone receptor superfamily (SHRS) that block epidermal ODC induction must be broadened to include the vitamin D_3 derivative MC903 and estriol. Our finding that MC903 was not capable of reducing lesional ODC levels, however, is probably due to a methodological problem. Instead of an at random designation of all biopsy sites at the beginning of the trial, those at the end of the trial were generally taken from small remaining psoriatic lesions at the periphery of the original plaque. The clinical relevance of our surprising finding with estriol, i.e. a pronounced inhibition of epidermal ODC induction in the range of a potent corticosteroid, will hopefully become clear in the near future.

In the Introduction we asked the question whether ODC could be used as a marker enzyme to test (new) antipsoriatic drugs. The answer has to be "yes", with the proviso that failure to block epidermal ODC induction does not necessarily exclude antipsoriatic potential. UVB and dithranol (unpublished data) induce ODC themselves, coal tar and cyclosporin A do not influence ODC induction and several ligands of the SHRS are potent inhibitors of ODC induction. These in the first instance conflicting data may well be explained by a vicious circle between the epidermis and the immune system; both stimulate/induce each other and it does not matter where in the circle an antipsoriatic therapy interferes in this process.

6.2 Signal transduction pathways mediated by inflammatory stimuli: current concepts

Combining the data described in this thesis with the facts that in the meantime have become available in the literature, gives the picture of the pathways involved in ODC induction as shown in Fig. 2, which differs from the pathway indicated in Fig. 6 of the Introduction. However, before explaining those routes in greater detail, the general overview will be enhanced by a re-evaluation of Fig. 2 in chapter I. In particular, it is now possible to suggest a mechanism for the "common pathway", regulating both proliferation, differentiation and inflammation after several kinds of "traumatizing " induction stimuli: the TGFα autocrine loop (Fig. 1).

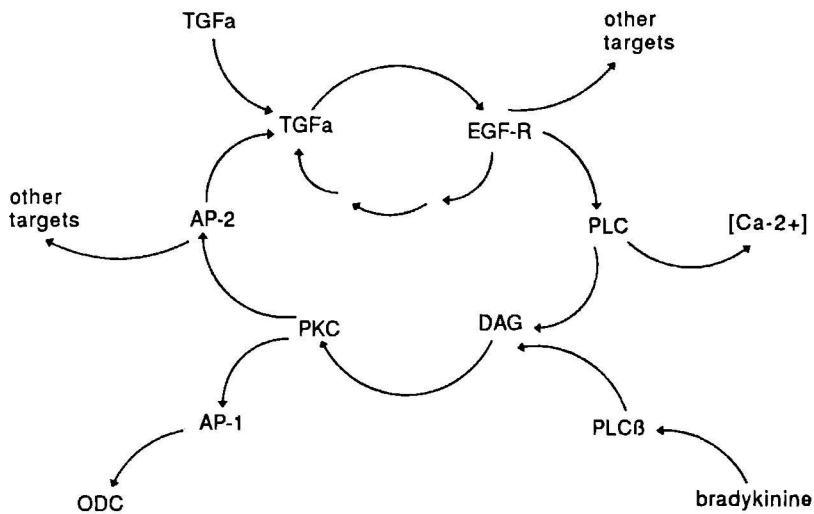

Fig. 1

When epidermal cells are activated (for example by TGFα in an autocrine or paracrine fashion), this will finally result in secretion of TGFα by these cells themselves and this vicious circle is known as the TGFα autocrine loop.[17] The loop is normally quiescent and becomes active only after an incoming "trauma" signal. This may be bradykinin in plasma,[18] but other cytokines including TNFα, IFNγ and IL8 have recently been shown to induce TGFα expression in organ cultures of human skin.[19] Several out-going signals further influence proliferation, inflammation and differentiation. If the loop gain is less than unity, the signal will eventually die out, and the skin will return to normal. If in the near future this rather hypothetical concept should prove to be correct, it would be logical to assume that psoriasis is characterized by an "intrinsic error" in the loop gain itself. Investigating the validity of this concept has just started in our laboratories.

Our current view of the transduction pathway of epidermal ODC induction is given in Fig. 2. The steps between stimulation of the EGF-R and activation of PKC have remained similar, but the following events have changed as result of new experimental data (for a review, see the special issue of Trends in Biochemical Sciences, Volume 17 No. 10, 1992, called 'Signal Transduction : Crosstalk'). In a simplified form, activated PKC stimulates the transcription factor AP-1, a c-jun/c-fos heterodimer,[20] that induces ODC transcription.[21] Activated AP-1 blocks the DNA-binding domain of the activated corticosteroid receptor (CS-R) and the reverse is also true: an explanation why corticosteroids reduce epidermal ODC induction.[22] Whether the other members of the SHRS have a same mode of action remains to be proven, but seems logical according to our findings.[23] The activated CS-R is a transcription factor for lipocortin (LC), that blocks

activity of PLA_2. However, phosphorylation of LC by PKC liberates PLA_2, hence the release of AA and its products will follow.[24] This explains why PGs and LTs are closely linked to, although not essential in the pathway of epidermal ODC induction, as shown in chapter II and III.

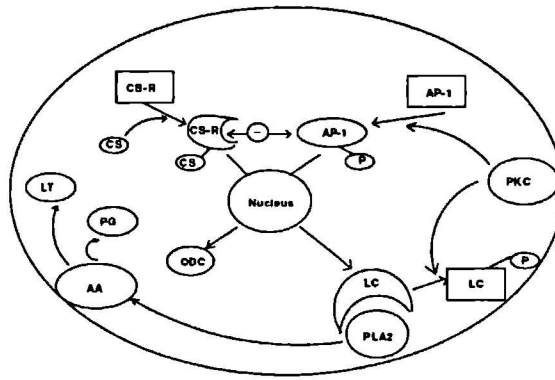

Fig. 2

6.3 Relevance of ODC in the future

"(...) Effective antipsoriatic agents (...) have also been found to reduce epidermal polyamine synthesis. Inhibition of polyamine synthesis may prevent growth in several experimental systems, and this inhibition is reversed by adding back polyamines. The inhibitor of polyamine synthesis, methylglyoxal-*bis*(guanylhydrazone) was also found to have a slight effect in controlling psoriatic lesions. (...) Further work is needed in this important area. (...)" [Camp RDR. Psoriasis. In: RH Champion, JL Burton, FJG Ebling

(eds.) *Textbook of Dermatology* (5th edition). Blackwell Sc Publ, Oxford 1992; 1399-1400]

"(...) the inhibition of polyamine production is and remains a feasible therapeutic approach in diverse areas. The reasons for the disappointing results in clinical cancer chemotherapy and the slow progress of the treatment of human hyperproliferative skin diseases with inhibitors of ODC (...) are merely technical ones. These include dosing of the drugs, cellular compensatory mechanisms triggered in response to polyamine depletion, galenic forms to be used topically, and selection of the right partner for combined regimens, just to mention a few. (...) What is the reason for the extraordinary sensitivity of trypanosomes (parasites) to difluoromethyl-ornithine (a direct ODC inhibitor) not only under experimental conditions, but also in clinical settings? A proper answer to this question may solve everything: Isn't a cancer cell also a parasite? (...)" [Jänne J, Alhonen-Hongisto L. Inhibitors of ornithine decarboxylase: biochemistry and applications. In: SI Hayashi (ed.) *Ornithine decarboxylase: biology, enzymology and molecular genetics*. Pergamon Press NY 1989; 77-78]

According to these relatively recent quotations, the relevance of epidermal ODC activity to psoriasis remains clear in the future. It seems of interest (both from a dermatological and oncological point of view) to study the influence of gut-decontamination and food-intake on ODC induction in "non-vascularized" epidermis. Do alterations in polyamine-uptake explain inter- and intra-individual differences in the level of ODC induction in human skin? Does polyamine-uptake modify expression of hyperproliferative skin diseases?

Clinical and laboratory studies to elucidate these questions seem worthwhile.

Looking at Figure I, ODC can be an indirect tool in verifying the mechanism of the TGFα loop (stimulation of cultured keratinocytes that have been arrested in G_0-phase) and elucidating the pathways for 'on' switches of vital importance (bradykinin, TNFα, IL8, amphiregulin,[25] etc.). Although, in fact, both require measurement of TGFα itself.

The interpretations of our results with sphingosine, tannic acid and estriol will certainly improve when data regarding their influence on ODC induction in cultured keratinocytes become available. This applied work is then easily extended by screening specific inhibitors of the key-enzymes in the TGFα loop, such as serine/threonine kinase inhibitors, tyrosine kinase inhibitors and receptor-antagonizing oligopeptides. Studies on the efficacy of these inhibitors will provide the ultimate reality testing of the pathogenetic relevance of TGFα associated processes in psoriasis. Thus, ODC studies can provide first indications for potent anti-inflammatory, cytostatic or anti-psoriatic agents, which may become available in the future: Isn't a psoriatic cell also a parasite?

REFERENCES

1 van Erp PEJ, Groenendal H, Bauer FW, Rijzewijk JJ. Growth fraction in epidermal skin disorders determined by the monoclonal antibody Ki-67. J Invest Dermatol 89: 313, 1987

2 Rijzewijk JJ, van Erp PEJ, Bauer FW. Two binding sites for Ki-67 related to quiescent and cycling cells in human epidermis. Acta Dermato Ven 69: 512-515, 1989

3 de Mare S, van Erp PEJ, Ramaekers FCS, van de Kerkhof PCM. Flow cytometric quantification of human epidermal cells expressing keratin 16 in vivo after standardized trauma. Arch Derm Res 282: 126-130,

1990

4 Mansbridge JN, Knapp AM. Changes in keratinocyte maturation during wound healing. J Invest Dermatol 89: 253-263, 1987

5 Pegg AE. Recent advances in the biochemistry of polyamines in eukaryotes. Biochem J 234: 249-262, 1986

6 Piacentini M, Farrace MG, Imparato M et al. Polyamine-dependent post-translational modification of proteins in differentiating mouse epidermal cells. J Invest Dermatol 94: 694-699, 1990

7 Fuchs E. Epidermal differentiation: the bare essentials. J Cell Biol 111: 2807-2814, 1990

8 Folk JE, Park MH, Chung SI et al. Polyamines as physiological substrates for transglutaminase. J Biol Chem 255: 3695-2700, 1980

9 Heby O. Role of polyamines in the control of cell proliferation and differentiation. Differentiation 19: 1-20, 1981

10 Bohm S, Berghard A, Pereswetoff-Morath C, Toftgard R. Isolation and characterization of complementary DNA clones corresponding to genes induced in mouse epidermis in vivo by tumor promoters. Cancer Res 50: 1626-1633, 1990

11 Fukuda M, Kono T, Ishii M et al. Increased expression of the ornithine decarboxylase gene in mouse skin by ultraviolet light: detection by in situ hybridization technique. Arch Dermatol Res 282: 487-489, 1990

12 Kono T, Fukuda M, Ishii M et al. Detection of ornithine decarboxylase gene expression in TPA-treated mouse skin using in situ hybridization. Acta Derm Venereol 71: 104-107, 1991

13 Panet R, Snijder D, Atlan H. Amiloride added together with bumetanide completely blocks mouse 3T3-cell exit from G0/G1-phase and entry into S-phase. Biochem J 239: 745-750, 1986

14 Sunkara PS, Baylin SB, Luk GD. Inhibitors of polyamine biosynthesis: cellular and in vivo effects on tumor proliferation . In: PP McCann, AE Pegg and A Sjoerdsma, eds. Inhibition of polyamine metabolism. Biological significance and basis for new therapies. Orlando (FL), Academic Press 1987; 121-140

15 Hessels J, Kingma AW, Ferwerda H et al. Microbial flora in the gastrointestinal tract abolishes cytostatic effects of α-difluoromethylornithine in vivo. Int J Cancer 43: 1155-1164, 1989

16 Verma AK, Pong R-C, Erickson D. Involvement of protein kinase C activation in ornithine decarboxylase

gene expression in primary culture of newborn mouse epidermal cells and in skin tumor promotion by TPA. Cancer Res 46: 6149-6155, 1986

17 Lee SW, Morhenn VB, Ilnicka M et al. Autocrine stimulation of interleukin 1α and transforming growth factor α production in human keratinocytes and its antagonism by glucocorticoids. J Invest Dermatol 97: 106-110, 1991

18 Talwar HS, Fisher GJ, Voorhees JJ. Bradykinin induces phosphoinositide turnover, 1,2-diglyceride formation and growth in cultured adult human keratinocytes. J Invest Dermatol 95: 705-710, 1990

19 Valyi-Nagy I, Jensen PJ, Albelda SM, Rodeck U. Cytokine-induced expression of transforming growth factorα and the epidermal growth factor receptor in neonatal skin explants. J Invest Dermatol 99: 350-356, 1992

20 Boyle WJ, Smeal T, Defize LHK et al. Activation of protein kinase C decreases phosphorylation of c-jun at sites that negatively regulate its DNA-binding activity. Cell 64: 573-584, 1991

21 Crabtree GR. Contingent genetic regulatory events in T lymphocyte activation. Science 243: 355-361, 1989

22 Yang-Yen H-F, Chambard J-C, Sun Y-L et al. Transcriptional interference between c-jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. Cell 62: 1205-1215, 1990

23 Schüle R, Umesono K, Mangelsdorf DJ et al. Jun-Fos and receptors for vitamins A and D recognize a common response element in the human osteocalcin gene. Cell 61: 497-504, 1990

24 Flower RJ. The mediators of steroid action. Nature 320: 20, 1986

25 Cook PW, Pittelkow MR, Keeble WW et al. Amphiregulin messenger RNA is elevated in psoriatic epidermis and gastrointestinal carcinomas. Cancer Res 52: 3224-3227, 1992

26 Karin M. The AP-1 complex and its role in transcriptional control by protein kinase C. In: *The hormonal control of gene transcription*, P Cohen, JG Foulkes, eds. Elsevier, Amsterdam 1991; 235-253

SUMMARY

In the Introduction (chapter I) a description of epidermal proliferation, inflammation and differentiation is presented and the close relations between these processes are discussed. All three are disturbed in the skin disease psoriasis, in a way that looks very similar to the response to an acute trauma. Ornithine decarboxylase (ODC) is closely involved in DNA replication and has been extensively investigated in rodent skin, but human data regarding epidermal ODC induction pathways are scarce. Therefore, in this thesis stripping of the stratum corneum and irradiation with UV-B are used as models for standardized trauma and the level of ODC induction is used as a marker for epidermal proliferation in humans *in vivo*.

Chapter II provides the basis for this thesis. This includes (I) a complete description of the methods used, (II) an ODC induction curve following epidermal stripping of healthy volunteers, (III) comparison of this curve with the ODC induction curve in psoriatic uninvolved skin and (IV) the influence of a topical corticosteroid (an indirect inhibitor of arachidonic acid) and oral indomethacin (a cyclooxygenase inhibitor) on this induction process. The results demonstrate an ODC peak after 8 h, no difference between healthy and psoriatic subjects, a marked inhibition by the topical corticosteroid, but no effect of oral indomethacin.

In chapter III the influence of prostaglandins and leukotrienes is further investigated,

since cyclooxygenase- and/or lipoxygenase-inhibitors have been claimed to block ODC induction in rodent skin. Topical Elmetacin^{R} (a cyclooxygenase inhibitor) and topical Dignodolin^{R} (which inhibits both cyclooxygenase and lipoxygenase) failed to show any inhibitory effect on epidermal ODC induction following either tape stripping or UV-B irradiation. This chapter excludes a role for eicosanoids in induction of ODC in human epidermis and indicates the danger of extrapolating rodent data to the human situation.

Chapter IV reports the effects of two previously uninvestigated ligands of the steroid hormone receptor superfamily on epidermal ODC induction, calcipotriol and estriol. Their inhibitory capacity proved to be in line with other members, namely corticosteroids and retinoic acid. Speculations regarding these findings become attractive, since corticosteroids, retinoic acid and recently also calcipotriol (as shown in paragraph 4.1) are all used as potent antipsoriatic drugs.

In chapter V the line of chapter IV is continued by asking whether ODC can be used as marker-enzyme to test (new) antipsoriatic therapies. Topical sphingosine (a protein kinase C inhibitor), isoquinoline (a component of the antipsoriatic agent coal tar), tannic acid (an inhibitor of ODC induction in mouse skin) and oral cyclosporin A (an immunosuppressive drug) were all tested for their capacity to block epidermal ODC induction and their efficacy in reducing the psoriatic plaque. Our results indicate that ODC can, indeed, be a useful marker, with the proviso that failure to block epidermal ODC induction does not necessarily exclude antipsoriatic potential.

Chapter VI reviews the major conclusions in the light of current concepts in signal transduction pathways. Two positive feedback loops are discussed. First, the vicious circle between the epidermis and the immune system results in a psoriatic plaque; both stimulate or induce each other, and an antipsoriatic drug may interfere with this cycle at various targets (chapters IV and V). Secondly, the epidermal TGFα autocrine loop may function as a common pathway, with input-signals from the epidermis and the immune system, and output-signals, controlling proliferation, inflammation and differentiation (i.e. ODC, as shown in chapters I and II). These findings support the hypothesis that psoriasis may be characterized by an "intrinsic error" in the TGFα loop gain.

SAMENVATTING (Dutch translation)

In de Introductie (hoofdstuk I) worden epidermale proliferatie, inflammatie en differentiatie beschreven en wordt de hechte relatie tussen deze processen besproken. Alle drie verlopen gestoord in de huidziekte psoriasis en wel op een wijze die sterke overeenkomsten vertoont met de reactie op een acuut trauma. Ornithine decarboxylase (ODC) is nauw betrokken bij de DNA-verdubbeling en is uitgebreid onderzocht in de huid van dieren, maar menselijke gegevens over de epidermale ODC inductie cascade zijn schaars. Daarom worden in dit proefschrift strippen van het stratum corneum en bestraling met UV-B benut als modellen voor een gestandaardiseerd trauma en wordt het niveau van ODC inductie gebruikt als een kenmerk voor humane epidermale proliferatie *in vivo*.

Hoofdstuk II levert de basis voor deze thesis. Dit omvat (I) een volledige beschrijving van de gebruikte methodologie, (II) een ODC inductie curve na epidermaal strippen van gezonde vrijwilligers, (III) een vergelijking van deze curve met de ODC inductie curve in de klinisch niet aangedane psoriatische huid en (IV) de invloed van een locaal corticosteroïd (een indirecte remmer van arachidonzuur) en oraal indomethacine (een cyclo-oxygenase remmer) op dit inductieproces. De resultaten laten een ODC piek na 8 uur zien, geen verschil tussen gezonde en psoriatische personen, een duidelijke remming door het locale corticosteroïd, maar geen effect van oraal indomethacine.

In hoofdstuk III wordt de invloed van prostaglandines en leukotriënes verder onderzocht, aangezien cyclo-oxygenase- en/of lipoxygenase-remmers de ODC inductie in de huid van muizen en ratten zouden blokkeren. Locaal Elmetacin^{R} (een cyclo-oxygenase remmer) en locaal Dignodolin^{R} (zowel een lipoxygenase- als cyclo-oxygenase-remmer) lieten absoluut geen blokkade zien van de epidermale ODC inductie, na tape strippen noch na UV-B bestraling. Dit hoofdstuk sluit een rol voor eicosanoïden in de ODC inductie in de menselijke huid uit en illustreert het gevaar van extrapoleren van dierlijke gegevens naar de humane situatie.

Hoofdstuk IV rapporteert over de effecten van twee niet eerder onderzochte leden van de steroïd hormoon receptor superfamilie op de epidermale ODC inductie, calcipotriol en estriol. Hun remmende eigenschappen bleken overeen te komen met twee andere leden, namelijk corticosteroïden en retinoïnezuur. Het is aantrekkelijk om over deze bevindingen te speculeren, aangezien corticosteroïden, retinoïnezuur en sinds kort eveneens calcipotriol (zoals wordt aangetoond in paragraaf 4.1) allen worden aangewend als krachtige antipsoriatische medicijnen.

In hoofdstuk V wordt de lijn in hoofdstuk IV gecontinueerd door te vragen of ODC kan worden gebruikt als "marker"-enzym voor het testen van (nieuwe) antipsoriatische therapieën. Topisch sphingosine (een proteine kinase C remmer), isoquinoline (een grondstof in het antipsoriatische middel koolteer), tanninezuur (een remmer van de ODC inductie in muizehuid) en oraal cyclosporine A (een immunosuppressief geneesmiddel)

werden allemaal getest op hun vermogen om de epidermale ODC inductie te blokkeren en voor hun effectiviteit in het reduceren van de psoriatische laesie. De resultaten geven een positief antwoord op de vraag of ODC als "marker"-enzym kan worden benut, zij het met de opmerking dat falen in het remmen van de epidermale ODC inductie niet per sé een antipsoriatisch vermogen uitsluit.

Hoofdstuk 6 bespreekt de voornaamste conclusies in het licht van de nieuwste inzichten in de cascades voor de overdracht van signalen. Twee positieve terugkoppelings-kringlopen worden besproken. Ten eerste resulteert de vicieuze cirkel tussen de epidermis en het immuunsysteem in een psoriatische laesie; beide stimuleren c.q. induceren elkaar en een antipsoriatisch medicijn kan op verschillende plaatsen in deze cirkel aangrijpen (hoofdstuk IV en V). Ten tweede kan de epidermale autocriene TGFα kringloop als een algemene centrale cascade fungeren, met binnenkomende signalen uit de epidermis en het immuunsysteem en met uitgaande signalen die proliferatie, inflammatie en differentiatie reguleren (bijv. ODC, zoals beschreven in hoofdstuk I en II). Deze bevindingen ondersteunen de hypothese dat psoriasis veroorzaakt kan worden door een intrinsieke fout in de "terugkoppelingsfactor" van de TGFα kringloop.

DANKWOORD

Dit dankwoord richt ik allereerst aan mijn ouders, die op velerlei wijze mijn (pre-doctorale) studie hebben ondersteund. Prof. Peter van de Kerkhof en prof. Paul Mier wil ik danken voor het feit, dat zij het uitvoeren van de in dit proefschrift beschreven onderzoeken financieel en zowel theoretisch als praktisch mogelijk hebben gemaakt. Joost Schalkwijk voor zijn sympathieke en kanttekening-rijke overname van het co-promotorschap. Candida van Hooijdonk voor het geduldig aanleren van de basistechnieken. Gijs de Jongh voor de statistische ondersteuning. Mieke Bergers voor het feit, dat ik geen betere kamergenote had kunnen treffen. Hans Alkemade voor zijn komische opmerkingen bij al mijn doen en laten. Piet van Erp en Zamira Arts voor hun hulp bij de lay-out van dit proefschrift; ik doe mijn voorletters nu eer aan. Bea Lintsen, Conrad Glade en Benno Pennings voor de leuke en vruchtbare samenwerking tijdens hun wetenschappelijke stage; dankzij jullie konden zowel het onderzoek als de klinische trials steeds doorgaan. Alle participerende gezonde vrijwilligers en psoriasispatienten voor hun stukjes huid, die dit *in vivo* onderzoek mogelijk hebben gemaakt. Renée Velde voor het ontwerpen van de voorkant van deze thesis. Arthur Gieles en Els van de Molen voor hun zware bijbaantje als paranymph. Luitpold Werke GmbH, LEO Pharmaceutical Products BV, Sandoz BV, Essex BV en Glaxo BV voor de financiële ondersteuning van de druk van het proefschrift. En last but not least Carla Bles voor de persoonlijke ondersteuning.

CURRICULUM VITAE

W. Peter Arnold werd op 3 december 1961 geboren in Arnhem. In 1981 behaalde hij het einddiploma VWO op het Christelijk Lyceum Veenendaal. Van 1981 tot 1990 studeerde hij geneeskunde aan de Katholieke Universiteit Nijmegen. In deze periode werkte hij eveneens een half jaar als verpleegkundige en werden twee studie-reizen naar Pakistan gemaakt, alwaar hij o.a. onderzoek deed naar her-infecties met Giardia Lamblia in een "gesloten" derde-wereld-gemeenschap.

Tijdens de wetenschappelijke stage op de afdeling Dermatologie van het Academisch Ziekenhuis Nijmegen (AZN) werd een begin gemaakt met het onderzoek dat aan deze dissertatie ten grondslag ligt. In de periode van maart 1990 tot december 1992 heeft hij als wetenschappelijk medewerker o.a. het in deze thesis beschreven onderzoek uitgevoerd, verwerkt en gepubliceerd. Tevens werkte hij 4 maanden als AGNIO op de polikliniek Dermatologie van het Twenteborg Ziekenhuis te Almelo. Per 1 december 1992 is hij in het AZN begonnen met zijn opleiding tot dermatoloog.